TUMOR NECROSIS FACTOR RECEPTOR RELEASING ENZYME

A new family of therapeutic agents
for treating arthritis
and other inflammatory conditions

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Exhibit 2

Ex cutive Summary

This presentation describes the commercial potential of a family of newly cloned enzymes that cause TNF receptors to be shed from the cell surface. TNF receptor releasing enzyme (TRRE) plays an important role in the natural control mechanism that regulates inflammation. The following pages illustrate some of the key properties of this enzyme family, and its potential for use in clinical therapy.

- TRRE activity was first isolated from the monocyte cell line THP-1. Subsequently, nine cDNA sequences encoding potential members of the TRRE family were cloned, expressed in bacteria, and evaluated by peptide cleavage assay (Section 3).

- Functional effectiveness of TRRE has been demonstrated with isolated cells and *in vivo*. Administration of exemplary TRRE clone MP8 increases the circulating level of shed TNF receptor by ~100-fold (Section 4).

- TRRE rescues experimental animals from a lethal dose of LPS in a dose-dependent fashion (Section 5). The therapeutic efficacy of TRRE is stable after extended storage, and persists in the circulation for at least three days.

- In a collagen-induced model of rheumatoid arthritis, prophylactic administration of TRRE inhibits joint swelling and development of arthritis. In established disease, administration of TRRE substantially reverses the extent of joint swelling (Section 6).

- TRRE also protects experimental animals against the pathology associated with carrageenan-induced edema, multiple sclerosis, and asthma (Section 7). It has been completely safe in all animal models tested.

There are several possible commercial embodiments for TRRE with considerable market potential (Section 2). Advantages over current anti-inflammatory agents include the following:

- TRRE has two modes of action: by releasing TNF receptor from the surface of effector cells, it both prevents signal transduction, and neutralizes any incoming ligand.

- TRRE cleaves the p55 and p75 isoforms of the TNF receptors, and the IL-6 receptor, thus inhibiting the inflammatory process through several important pathways.

- Each TRRE enzyme inactivates a number of TNF receptors, accomplishing in catalytic amounts what compounds like *Enbrel* ® accomplish in stochiometric amounts. Furthermore, it can readily produced by bacterial expression. These features imply facilitated commercial production and lower cost per unit dose.

Exemplary TRRE clone MP8 is a new metalloprotease with a novel sequence. Meyer Pharmaceuticals is obtaining patents worldwide with broad coverage for the commercially promising aspects of the TRRE family (Section 8). Corporate partnerships are now being sought to accelerate development and clinical testing.

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MP8 protects against pathological features of edema, multiple sclerosis, and asthma

8 Patent Protection

Patents are granted or pending worldwide for isolated TRRE and the cDNA sequence

Version 3.2

Mey r Pharmaceuticals

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Meyer Pharmaceuticals is a biotechnology venture company with head office and laboratories located in Orange County, California. It conducts developmental research and sponsors early phase clinical trials for new technologies with clinical promise.

Central interests are cancer immunotherapy, and a new family of proteolytic enzymes that cleave cytokine receptors and modulate the inflammatory response (the subject of this presentation). Intellectual property licensed and owned by the company covers all the promising commercial embodiments.

History of the Company

Meyer Pharmaceuticals is privately held and financed by Robert E. Meyer. It is part of a diversified portfolio of commercial ventures in several growing areas, including real estate, agricultural infrastructure, merchant banking, and biopharmaceuticals.

Meyer Pharmaceuticals was created as a way of allowing ill patients to benefit from important research work being conducted at the University of California at Irvine. The collaboration began in 1995 when the Robert Meyer Trust was seeking to make a philanthropic donation to advance the course of medical research in immunology.

Particularly compelling was work being done at the University on the immunology and molecular biology of cancer and autoimmune conditions. Dr. Gale A. Granger had a long-established reputation for discovery of TNF-β and soluble TNF receptor.

From the initial meetings with the scientists, two conclusions were reached. First, there was considerable potential for some of this technology in the treatment of human disease — new strategies for eliciting an anti-tumor response in cancer patients, and work being done relating to TNF receptor shedding in inflammation. However, the financial and structural support for taking these discoveries forward for clinical development was not in place.

It was decided that the best way to realize the potential of this work was to create a new biotechnology venture. The new company would undertake preclinical research needed to characterize the discoveries made at the university, and take the projects forward for commercial partnering.

Accordingly, Meyer Pharmaceuticals was created in 1996, and opened research facilities in Irvine, California. Tetsuya Gatanaga (Dr. Granger's chief collaborator on the TNF project) was recruited to the new company to direct the scientific research. Since then, the company has chosen a path of preclinical research and early-stage clinical trials to test and optimize aspects important for rapid commercialization.

Mission

Meyer Pharmaceuticals is a Service Mark for Pharmaceutical Research. The mission is to transition promising academic research into commercial therapeutic products, for the benefit of patients afflicted by debilitating clinical conditions. Meyer Pharmaceuticals® provides a drug screening and assay service, formulates pharmaceutical compositions, conducts preclinical research to evaluate commercial potential, and initiates early phase clinical trials.

Executive and Scientists

Michael F. O'Neill is the President and Chief Executive Officer of Meyer Pharmaceuticals. He played the central role in forming the company and acquiring core technologies from the University of California. He continues to actively manage the company's scientific research programs, corporate development, and intellectual property. Mr. O'Neill also plays prominent roles in other Meyer companies, and currently serves as Vice Chairman of the Meyer Group Executive Committee. He holds a Juris Doctor degree from the University of California Berkley Boalt Hall School of Law, and is a licensed attorney. Before joining Robert Meyer in 1991, Mr. O'Neill spent time as a consultant with Touche-Ross (now Deloitte and Touche), and in private legal practice, when he founded and operated several private business ventures.

Tetsuya Gatanaga is the Executive Vice President and Chief Operating Officer of Meyer Pharmaceuticals. He received his Ph.D. for creating a new recombinant form of TNF. In 1989, Dr. Gatanaga came to the University of California at Irvine, and in collaboration with Gale A. Granger, was the first to isolate TNF blocking factor (soluble TNF receptor). He subsequently isolated the enzyme responsible for causing the release of cell-surface TNF receptor, which is the subject of this presentation. Dr. Gatanaga joined Meyer Pharmaceuticals when the company was first formed in 1996, and oversees all of the scientific research projects.

Ronald L. Niece is Director of Cytokine Research at Meyer Pharmaceuticals. He received his Ph.D. from University of Wisconsin in evolutionary genetics, and then provided the University and commercial ventures with fee-based resource capabilities for automated protein and nucleic acid chemistry. Dr. Niece was one of the founders of the Association of Biomolecular Resource Facilities. He joined Meyer Pharmaceuticals in 1999, and works exclusively on the preparation, characterization, and preclinical testing of TRRE.

Michael Schiff is Director of Intellectual Property for the company. He has a Ph.D. in immunology, and has been licensed before the U.S. Patent & Trademark Office since 1995. He shares his time as patent counsel for Meyer Pharmaceuticals and Geron Corporation.

Gary Firestein, M.D., Chief of Rheumatology, Allergy and Immunology, University of California at San Diego; and **Carl Ware, Ph.D.**, Head of Molecular Immunology, La Jolla Institute of Allergy & Immunology, provide advice on the TRRE project as members of Meyer Pharmaceuticals' Scientific Advisory Panel.

Patent Portfolio

Meyer Pharmaceuticals has a policy of aggressive patent prosecution to cover commercially promising technologies both in the U.S. and overseas. Patents are licensed exclusively from the University of California for discovery research, and owned by the company for inventions subsequently made in its own laboratories.

Number of Patents		Receptor protease technology	Other technologies
U.S.	Issued & Allowed	3	7
	Pending	5	4
Foreign	Granted	5	6
	Pending	25	18

Selected Examples		
US 5,837,233	Nov 17, 1998	Method for treating tumors
US 6,203,787	Mar 20, 2001	Treating tumors using implants comprising combinations of allogeneic cells
US 6,207,147	Mar 27, 2001	Cancer immunotherapy using tumor cells combined with mixed lymphocytes
US 6,277,368	Aug 21, 2001	Cancer immunotherapy using autologous tumor cells combined with cells expressing a membrane cytokine
US 6,368,593	Apr 9, 2002	Enhanced immunogenic cell populations using H2 receptor antagonists
US 6,569,664	May 27, 2003	Native TNF receptor releasing enzyme
US 6,573,062	June 3, 2003	Method for obtaining modulators of TNF receptor releasing enzyme
US 6,593,456	July 15, 2003	Tumor necrosis factor receptor releasing enzyme
EP 1037643 B1	Sept 27, 2000	Cancer immunotherapy using allostimulated cells in a multiple sequential implantation strategy
WO 99/58559	Nov 18, 1999	Factors affecting tumor necrosis factor receptor releasing activity
WO 00/03033	Jan 20, 2000	Use of fluorescent reagents in identification of cancerous cells and activated lymphocytes

Market pot ntial for TRRE

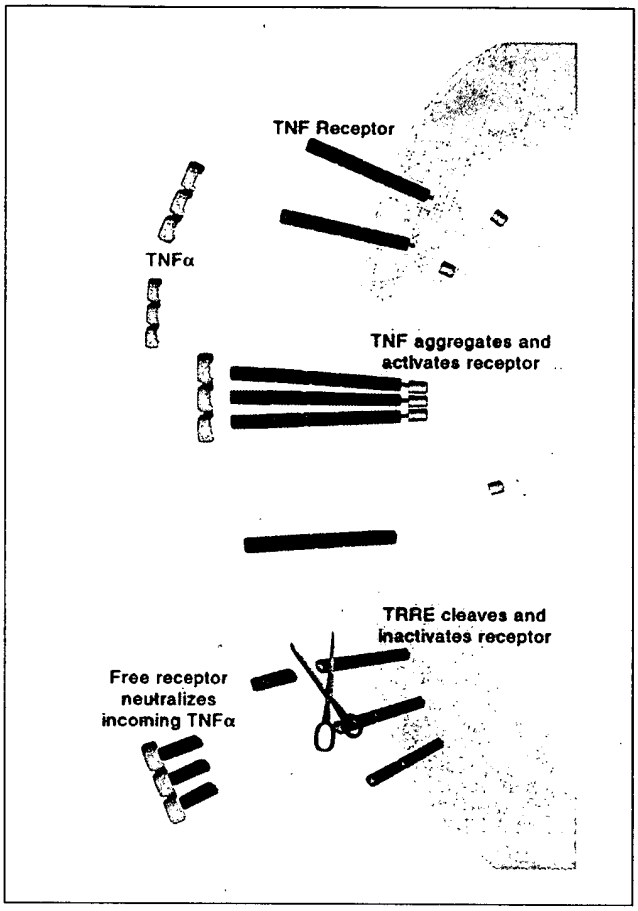
The market potential for TNF inhibitors in treating rheumatoid arthritis is estimated to exceed $20 billion annually in the U.S., with comparable potentials in both Europe and Asia. Other conditions with large patient populations (such as Crohn's disease, psoriasis, and ankylosing spondylitis) also respond to TNF inhibitors. This area may be the largest single market opportunity in the pharmaceutical field.

TRRE has a number of important advantages as a TNF inhibitor, giving it considerable potential to compete with existing products, and to expand the target market.

Role of TRRE in inflammatory conditions such as arthritis

TNF-α and lymphotoxin (TNF-β) are key cytokines that induce inflammation and cause cell apoptosis in conditions such as rheumatoid arthritis. They are produced by inducer cells and bind receptors on the surface of effector cells: the p55 TNF receptor (TNF-R1), and the p75 TNF receptor (TNF-R2). Either TNF ligand can cause the receptor to trimerize, which initiates a number of intracellular sequelae, potentially leading to apoptosis (programmed cell death).

Inflammation is upregulated in disease conditions by increased TNF production by inducer cells. Therapeutic compounds such as *Enbrel* ® work by capturing TNF-α before it gets to the effector cell. There is also a natural down-regulation of the inflammatory process, caused by shedding of the TNF Receptor from effector cells.



TRRE is a family of enzyme discovered by scientists at Meyer Pharmaceuticals that is responsible for shedding of both the p55 and p75 TNF receptor. Enhancing the activity of TRRE provides a new strategy for controlling inflammatory disease.

The TRRE family

This presentation provides data obtained using the cDNA clone designated MP8. In fact, nine different cDNA clones affecting TNF receptor shedding have been isolated and sequenced. MP8 and at least one of the other clones encode metalloproteases with specific TNF receptor cleaving activity. The rest may be involved in regulating the expression of TRRE activity in an important way.

Meyer Pharmaceuticals is actively seeking patent protection for the original TRRE isolate and all nine cDNA clones. In addition, research work is underway using a new strategy to isolate and characterize additional members of the TRRE family.

Commercial embodiments of TRRE

Since TRRE is the naturally occurring enzyme that causes receptor shedding, there are a number of potential industrial applications.

1. The lead embodiment is the use of TRRE protein (expressed from the cDNA clone) for the treatment of inflammatory conditions in which TNF is implicated. There are a number of possible indications with large markets — for which the prototype is rheumatoid arthritis. The data provided later in this presentation show that TRRE need not be given intravenously to obtain a therapeutic effect. Subcutaneous administration is just as effective, which means that the commercial product can be self-administered by the consumer. It should also be possible to formulate TRRE cDNA for genetic therapy of inflammatory conditions in a therapeutic vector.

2. Since TRRE is an enzyme, it can also be used as a screening agent for discovery of small molecule drugs that increase or decrease receptor shedding — thereby controlling the inflammatory process. It can also be used as part of a screening process to identify drugs that inhibit other enzymes involved in inflammation (such as TACE) without inhibiting TRRE.

3. There is also the potential to treat conditions with the opposite underlying defect — insufficient TNF signaling due to too much receptor shedding. It is known that both the p55 and p75 TNF receptors are shed in cancer (Grosen et al., Gynecol. Oncol. 50:68, 1993). This is presumably because cancer cells up-regulate TRRE in order to evade inflammatory attack. If this is correct, then antibody or antisense compounds directed at TRRE may have potential for treating cancer, and other conditions associated with increased TNF receptor cleavage. Measuring TRRE levels may also be a useful diagnostic method for determining cancer or monitoring treatment.

Market Size for Anti-Inflammatories (Embodiment 1)

The incidence of rheumatoid arthritis throughout the world is approximately one percent of the population. Methotrexate, still the mainstay of therapy, is successful in only about one third of those affected, as indicated by at least 50% improvement in composite assessment scales. There is also evidence from recent clinical trials that cytokine regulators provide patients with bone and cartilage protection, regardless of whether there is symptomatic improvement — suggesting that cytokine regulating agents should become the treatment of choice.

This puts the U.S. market for arthritis drugs such as TRRE at over 2 million patients. Assuming a retail cost of $10,000 per patient per year, the potential annual revenue is *over $20 billion* in the U.S. alone, just for treating arthritis. Market potential for Europe is comparable in size, and there are other markets in the Far East and Southern Hemisphere.

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TRRE also has the potential for use in treating other inflammatory conditions in which TNF is implicated — for example, other forms of arthritis, Crohn's disease, psoriasis, anklyosing spondylitis, multiple sclerosis, and asthma. These conditions affect many millions of people both in the U.S. and abroad. The market potential for new biological anti-inflammatory agents such as TRRE is considerable.

The established market of biological agents for treating arthritis

Small-molecule antirheumatic drugs such as methotrexate and sulfasalazine are insufficient to control inflammation in about two-thirds of arthritis patients. New biological agents developed in the last decade have proved to be effective for a majority of patients unresponsive to traditional drugs.

A leading recombinant protein for treating inflammatory conditions is *Enbrel* ® (Etanercept), marketed by Amgen Corp. It is a chimeric molecule comprising the extracellular portion of the human TNF receptor linked as a dimer to the IgG Fc region. The compound interferes with the binding of TNF to cell-surface TNF receptors — showing the importance of modulating the TNF pathway for clinical therapy of inflammatory conditions.

Enbrel ® is licensed in the U.S. for treatment of patients with moderate to severe rheumatoid arthritis, juvenile rheumatoid arthritis, and psoriatic arthritis. Approval is expected in 2003 for treating ankylosing spondylitis.

Sales of *Enbrel* ® were $750 million in 2001. Scaling up production to meet growing demand has been a challenge. The projected sales in the U.S. market for current indication is expected to reach $4 billion by 2005, just for current indications. The expected future revenue from *Enbrel* ® was key to the valuation of Immunex in its recent acquisition by Amgen Corporation for $10.4 billion.

Other biological agents currently licensed in the U.S. for treating arthritis are *Remicade* ® (Infliximab), a chimeric antibody that binds the TNF-α ligand; and *Kineret* ™ (Anakinra), a recombinant form of IL-1Ra, an antagonist of the interleukin-1 receptor. Sales of *Remicade* ® approximate those of *Enbrel* ®.

Important advantag s f TRRE clone MP8

Two modes of action

Cleavage of TNF receptors inhibits the TNF pathway in two ways: First, the receptor is removed from the membrane of the effector cell, so that it cannot participate in signal transduction. Second, the released ligand binding portion of the receptor neutralizes any incoming TNF ligand in a manner comparable with *Enbrel* ® and *Remicade* ®.

Effects on multiple pathways

TRRE specifically cleaves both isoforms of the TNF receptor, preferentially neutralizing the p55, which is primary isoform mediating inflammation. It also has cleaving activity for the IL-6 receptor, and receptors in other important cytokine pathways involved in inflammation.

Enzyme efficiency

Since TRRE is an enzyme, it differs from current anti-inflammatory agents. It has the potential to accomplish in catalytic amounts what receptor antagonists like *Enbrel* ® accomplish in stochiometric amounts. This means that a single molecule of TRRE should inactivate many TNF ligands and receptors, resulting in greater effect per molecule of administered drug. There are also regulatory advantages.

Natural compound

TRRE is a naturally occurring human protein that normally acts to regulate inflammation. This means should not be immunogenic. Furthermore, the TNF receptor it releases from the cell is an endogenous (non-recombinant) compound that neutralizes TNF ligand in a physiologically natural way.

(continued)

Important advantag s of TRRE clone MP8

Safety

TRRE shares with other specific biological agents the potential for a low side effect profile. No safety issues have arisen in 5 different animal disease models. Specificity of biological agents facilitates rapid completion of clinical trials.

Small size

Clone MP8 is a relatively small protein, causing greater effect per mass, while retaining the specificity and clinical benefit of biological agents. The small size also provides a range of options for clinical formulation, including intradermal delivery, which would allow administration close to an inflamed joint.

Long-lasting effects

Data in this report indicate that TNF receptor released by TRRE persist for days after administration. This means that administration of TRRE just once a week (or less) may be sufficient for a full therapeutic effect.

Synergistic potential

Since TRRE works by different mechanisms than currently established drugs, it has potential not just as an alternative — it may also improve the effect of other therapeutic agents, increasing the number of indications and market size.

Low cost of manufacture

TRRE can be produced by bacterial expression. It does not require glycosylation in mammalian cells like antibody products such as *Remicade* ®, or immunoglobulin derivatives such as *Enbrel* ®. The modest cost of production per dose will be an important competitive advantage.

Discovery and Activity M asurem nt for TRRE Clon MP8

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TRRE was first isolated from a monocyte cell line by a combination of protein separation techniques. Multiple cDNA sequences were cloned from a T cell expression library by transfection into cells engineered to express the TNF receptor. TRRE clone MP8 is a new metalloprotease with a novel sequence.

A rapid peptide cleavage assay has been developed for measuring TRRE activity. Clone MP8 cleaves both the p55 and p75 TNF receptor and the IL-6 receptor, but not control peptides. The assay can be used to quantitate TRRE activity during purification, and to screen potential small-molecule TRRE inhibitors or activators.

Isolation of TRRE from monocyte cell supernatant

Shedding of receptors for cytokines like TNF and IL-6 in inflammatory conditions has been known for over a decade. However, little progress was made towards characterizing and isolating the responsible protease, until Tetsuya Gatanaga and Gale Granger began their collaboration at UC Irvine.

First, an assay was designed to follow TRRE activity. COS cells were stably transfected to express the cDNA for the p75 receptor at a high level. When these cells were combined with supernatant from induced cells of the monocyte line THP-1, cleavage and release of the recombinantly expressed receptor was observed both by immunofluorescence analysis of receptor on the cell surface, and ELISA determination of released receptor in the supernatant.

This cell line provided a means for tracking TRRE activity during protein separation techniques. A combination of techniques was discovered that produced TRRE with a high degree of purity. First, 8 roller bottles of THP-1 cells were induced with phorbol myristate acetate (PMA), and the culture medium was harvested. Protein was precipitated with ammonium sulfate, dissolved, dialyzed, and loaded onto a DEAE-Sephadex® column. The column was eluted with a linear gradient of NaCl in Tris buffer, pH 8.0, and the fractions containing the highest TRRE specific activity were concentrated. The preparation was then electrophoresed on polyacrylamide under non-denaturing conditions, and the gel strips with highest specific activity were eluted and concentrated.

TRRE purified from THP-1 cells was found to cleave both the p55 and p75 TNF receptor. The activity requires presence of Ca^{++} and Zn^{++} in the solution, and is inhibited by EDTA. This indicates that TRRE is a metalloprotease. Secretion of TRRE is not blocked by inhibitors of protein synthesis, indicating that it is pre-formed inside the cell and released upon induction.

Cloning f cDNA ncoding TRRE activity

The cDNA for TRRE was obtained by transfecting the COS cells expressing TNF receptor with a cDNA expression library from Jurkat T cells, a cell line that expresses TRRE activity. The transfected cell population was cloned by limiting dilution, and the clones were tested for a capacity to cleave TNF receptor from the cell surface.

From the selected clones, the cDNA shuttle vector was reisolated, and transfected back into the COS cells. Three rounds of transfection and selection were performed, effectively screening over 10^6 clones. Nine candidates were isolated, sequenced, inserted in-frame into pAcGHLT-A baculovirus transfer vectors, and expressed in bacteria to provide batch preparations of the encoded protein.

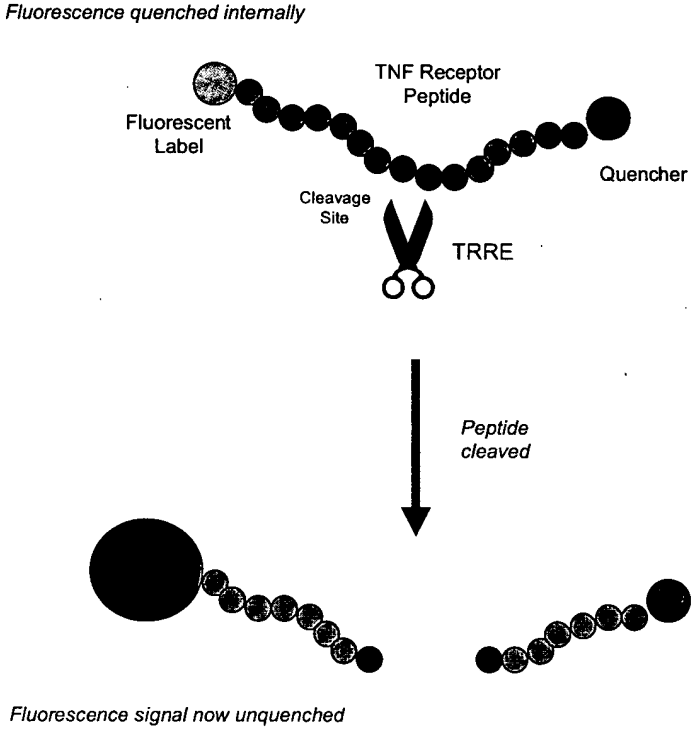
This strategy should select out cDNA clones that either have TRRE activity, or play a role in modulating TRRE activity in the transfected cells. Two of the isolated clones encode metalloproteases. Both of these clones have been fully sequenced, and both sequences were novel at the time the patent application was filed.

The data provided in this presentation were all obtained using the clone designated MP8.

For current production, the MP8 sequence has been cloned into a commercial expression vector. The sequence is expressed behind an N-terminal His Tag sequence followed by a thrombin site. The vector is expressed and purified at external facilities under contract from Meyer Pharmaceuticals. The protein extract is chromatographed on Q-Sepharose®, the peak is purified by fast-flow chromatography on Ni-NTA, and endotoxin levels are reduced on a Q-Sepharose® (HiTrap) column. Endotoxin level is measured using a chromogenic LAL assay, and TRRE activity is measured by fluorescence resonance energy transfer, as described on the next page.

Fluoresc nce Assay for TRRE activity

TRRE activity can rapidly be quantified by Fluorescence Resonance Energy Transfer (FRET). Peptides having the amino acid sequence of the TNF Receptor or other protein substrates are labeled at opposite ends with a fluorescence emitter and a fluorescence quencher. The peptide is then incubated with a source of TRRE, and fluorescence is measured. The quenching group normally absorbs fluorescence from the emitter. But enzymatic cleavage of the peptide decouples the quenching group, and fluorescence emission increases proportionally.



Peptides are labeled with the fluorescence emitter (Edans-▲) at the C-terminal, and the quenching hapten (Dabcyl-) at the N-terminal.

The assay is conducted in the presence of the metal cations Zn^{++} (0.05 mM) and Ca^{++} (2 mM), and corrected for cleavage in the presence of EDTA (20 mM). Dependence on divalent cations confirms that TRRE is a metalloprotease. The assay mixture also contains a cocktail of protease inhibitors and bovine albumin. Cleavage is measured as the net change in fluorescence emission after incubating TRRE with the peptide at 37°C for 3 hours.

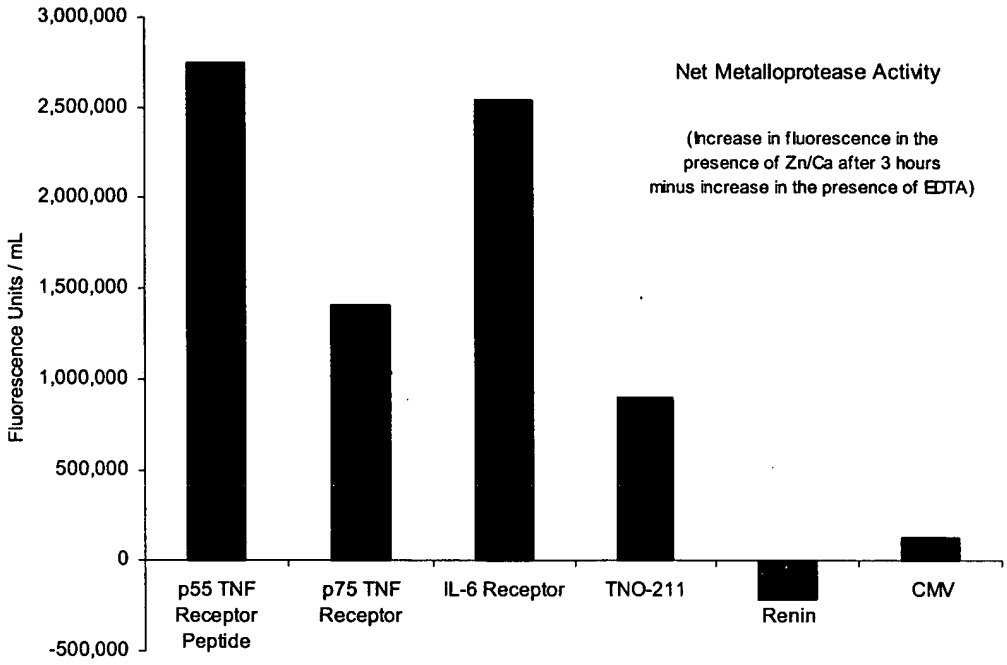
The peptides used in this assay are synthesized from the sequence of the proteolytic cleavage sites of the natural protein substrate.

TRRE substrate peptides:

p55 TNF Receptor	●-N-V-K-G-T-E-D-S-G-▲
p75 TNF Receptor	●-C-T-S-T-S-P-T-R-▲
IL-6 Receptor	●-A-N-A-T-S-L-P-▲

Other substrate peptides:

TNO-211 (matrix metalloprotease substrate)	●-γ-Abu-P-Q-G-L-E(▲)-A-K-NH_2
Renin aspartyl protease substrate	●-γ-Abu-I-H-P-F-H-L-V-I-H-T-▲
CMV serine protease substrate	●-R-G-V-V-N-A-S-S-R-L-A-▲

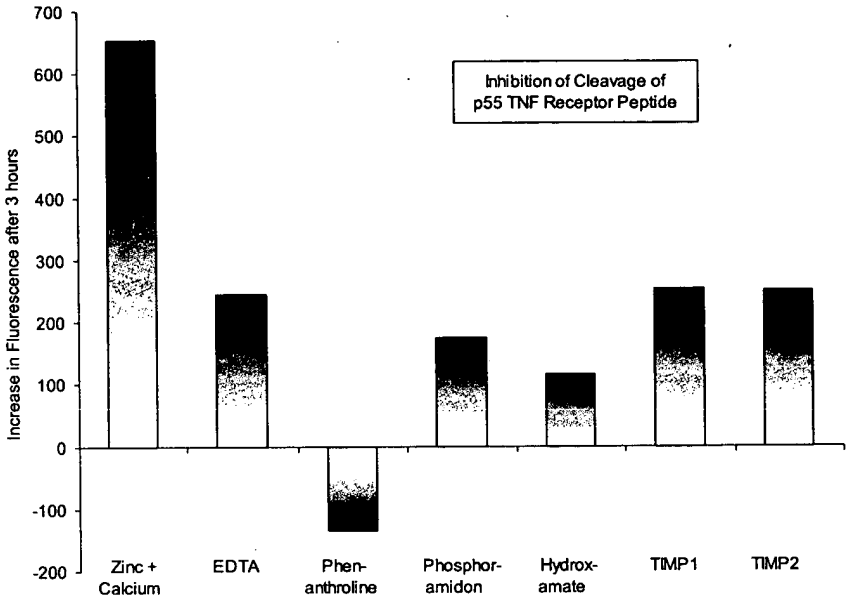


This graph shows results of a typical FRET assay for the purified TRRE clone MP8.

Enzyme activity is calculated as Fluorescence Units per mL, the activity that causes increase in fluorescence at 460 nm in the presence of Zn^{++} and Ca^{++}, corrected for the activity measured in the presence of EDTA.

MP8 specifically cleaves the peptides spanning the cleavage site of the p55 and p75 TNF receptor isotypes. MP8 also cleaves the IL-6 receptor peptide. Data published elsewhere show that metalloproteases ADAM-10 and MDC-9 do not efficiently cleave either the p55 or p75 TNF receptor.

This is the standard assay method at Meyer Pharmaceuticals for quantitation of TRRE activity during purification and preclinical assessment.



Use of FRET assay to screen small molecule inhibitors

This experiment illustrates the use of the FRET assay to assess potential inhibitors or activators of TRRE. The MP8 protein was combined with the inhibitor, added to the FRET peptide, and cleavage of the TNF p55 peptide was measured as increased fluorescence after 3 hours.

Inhibitors were as follows: 1,10 phenanthroline at 40 mM; phospharamidon at 500 μM; hydroxamate (Pharmingen) at 2 mM; TIMP-1 (Chemicon) at 2.5 μg/mL; TIMP-2 (Chemicon) at 2.5 μg/mL. Hydroxamate is a small molecule metalloprotease inhibitor. The TIMPs are naturally occurring tissue metalloprotease inhibitors.

All of these compounds inhibit the TRRE activity of MP8, compared with the activity in the presence of zinc and calcium alone.

TRRE caus s TNF rec ptor releas in vivo

When TRRE is incubated with cells that naturally express TNF receptor, the receptor is cleaved from the membrane and released. Administration of TRRE intravenously into test animals leads to release of both the p55 and p75 TNF receptor isotypes. This causes free receptor to accumulate in the circulation at a level over 100-fold above normal.

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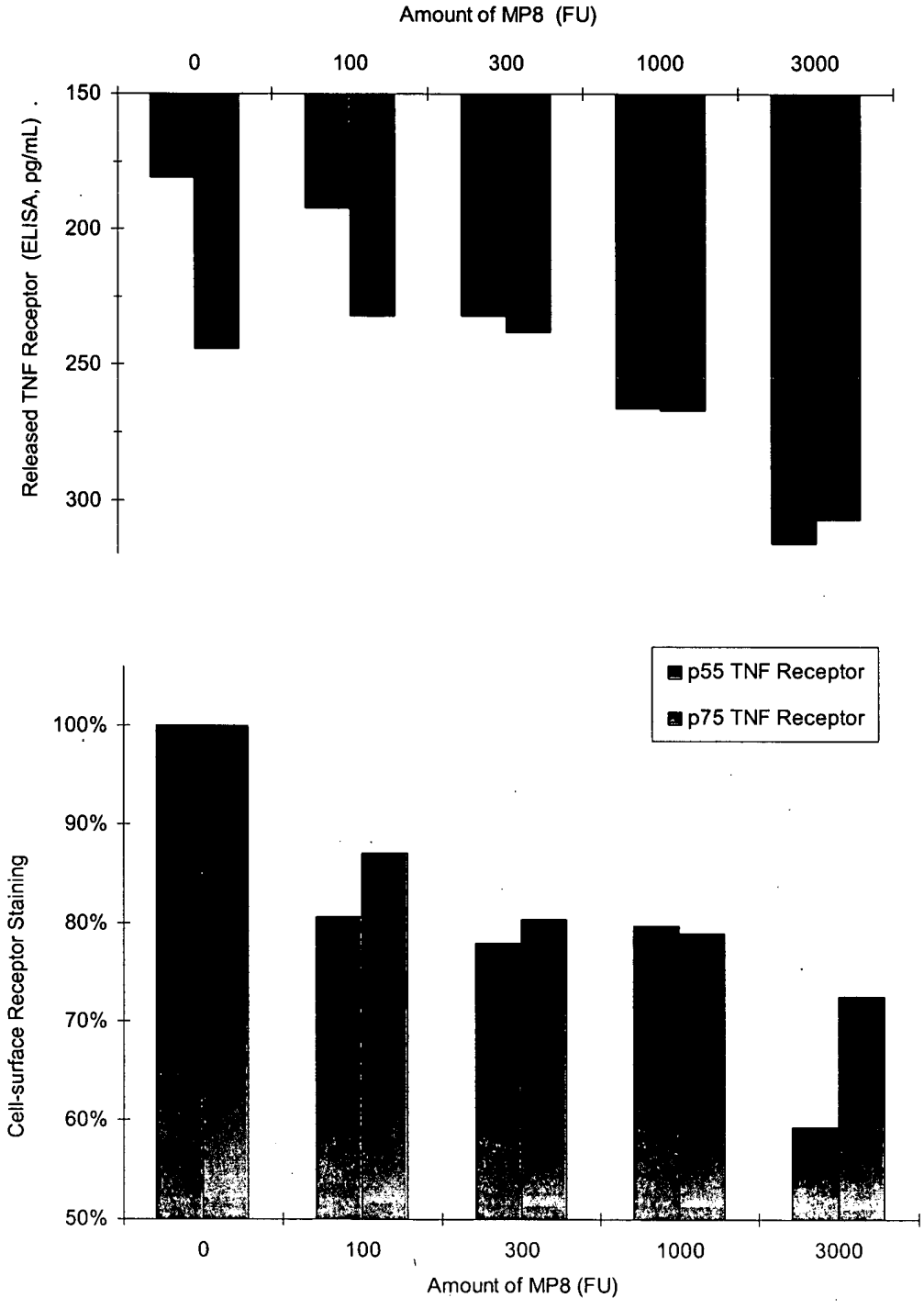
TRRE causes release of TNF receptor from THP-1 cells

To demonstrate TRRE clone MP8 is a protease capable of cleaving TNF receptors from cells, MP8 was incubated with THP-1 cells. This is a monocyte cell line that expresses both the p55 and p75 isotypes of the TNF receptor. After incubating for 45 minutes at 37°C, the cells were washed, immunostained for cell surface receptor, and counted by flow cytometry.

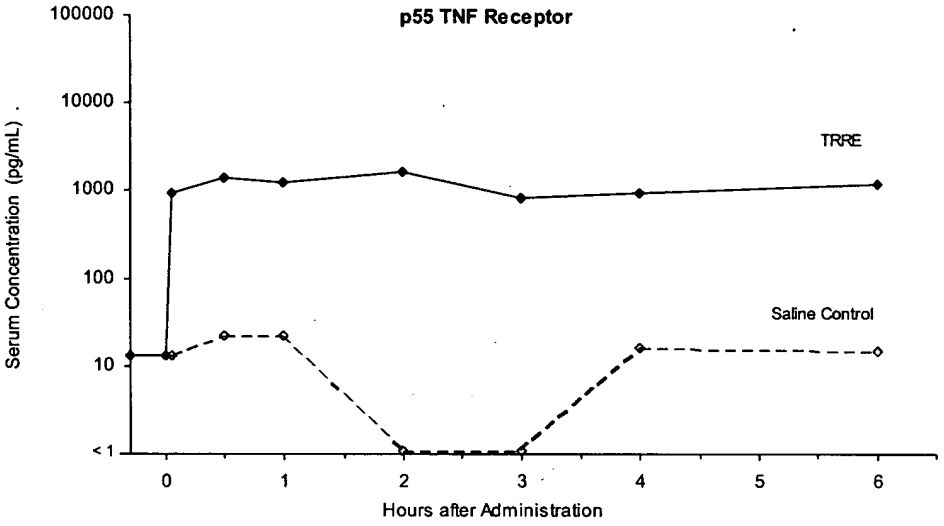
The data on the next page show that MP8 cleaves both the p55 and p75 TNF receptors from the surface of live cells. As a result, the receptors are shed and accumulate in the culture medium, as measured by enzyme immunoassay.

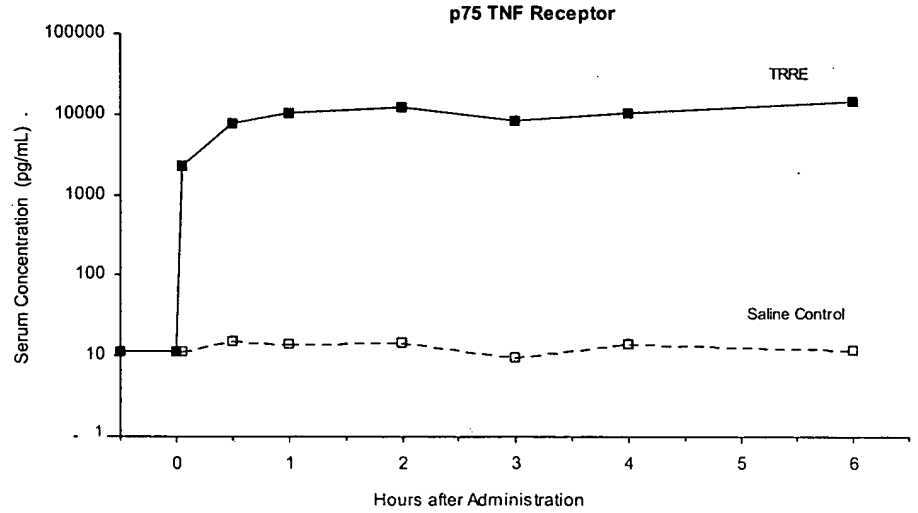

Amount of MP8 (FU)
0
100
300
1000
3000
150
200
250
300
Released TNF Receptor (ELISA, pg/mL)
p55 TNF Receptor
p75 TNF Receptor
100%
90%
80%
70%
60%
50%
Cell-surface Receptor Staining
0
100
300
1000
3000
Amount of MP8 (FU)

TNF increases circulating cytokin levels in mice

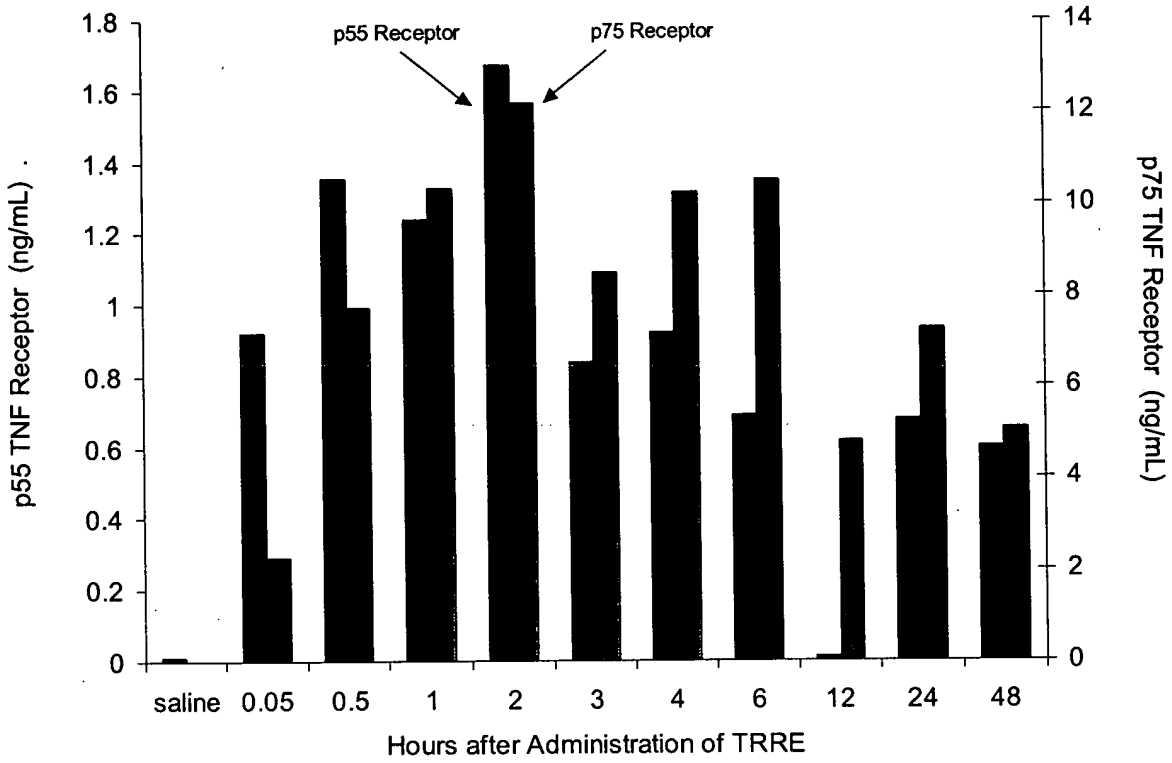
To determine if TRRE cleaves TNF receptors *in vivo*, 100,000 FU of purified MP8 was injected subcutaneously as a 500 µL bolus into female Balb/c mice. Control mice were injected with saline. Serum was sampled periodically, and tested for cytokine levels using ELISA kits from R&D System.

The data show that TRRE causes shedding of TNF receptor to an extent that causes it to accumulate in the circulation.





The graph below is a compilation of data from two experiments, showing the kinetics of receptor release over a 48 hour period.



These data have several important implications:

- Both the p55 and p75 TNF receptors are cleaved *in vivo* — meaning that signal transduction through either receptor will be affected.
- TRRE increases the level of circulating TNF receptor by about 100-fold. Administered TRRE should affect TNF signal transduction in two ways — by removing TNF receptor from the surface of inflammatory cells at the affected site, and creating an extracellular sink for TNF ligand.
- The effect of TRRE persists for at least 48 hours after administration — meaning that frequent dosing is not required.
- Human TRRE is capable of cleaving TNF receptors of other species. This validates use of the mouse as a model for studying the effects of TRRE as a therapeutic agent.

TRRE protects against s ptic shock

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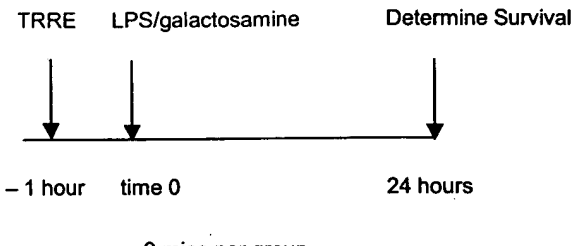
A classic model for determining effectiveness of agents against cytokine-mediated inflammation is endotoxin-induced septic shock. When tested in this model, TRRE was found to protect against septic shock in a dose-dependent fashion.

TRRE retains its full activity for more than a month's storage, both in terms of its proteolytic activity in the FRET assay, and its protective effect in the septic shock model. It is able to protect mice against LPS challenge for at least 3 days after administration — indicating that frequent dosing with TRRE is not required to have a therapeutically beneficial effect.

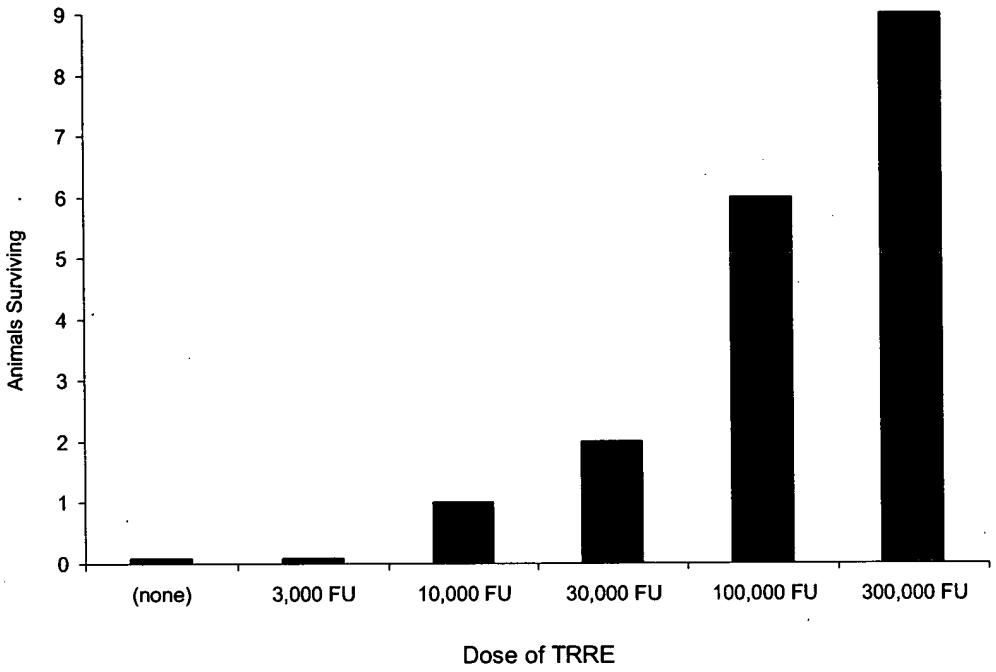
The septic shock model

In order to test the effectiveness of TRRE in septic shock, clone MP8 was purified under contract by Alliance Protein Laboratories from source material produced at Biosource International. The enzyme was purified using Nickel NTA column chromatography and Q-Sepharose® analytical column chromatography. Endotoxin level was reduced using Q-Sepharose®. Enzymatic activity was determined in the FRET assay, and endotoxin contamination was determined in a chromogenic LAL assay. Endotoxin was 0.16 µg per 300,000 fluorescence units of enzyme activity.

Procedure. Female Balb/c mice were randomized by weight into different treatment groups. Sepsis was induced by injecting 10 µg LPS and 7 mg galactosamine in the lateral or dorsal caudal vein. Some groups were pretreated TRRE as a protective agent against lethality of the LPS challenge.



9 mice per group



Dose Response

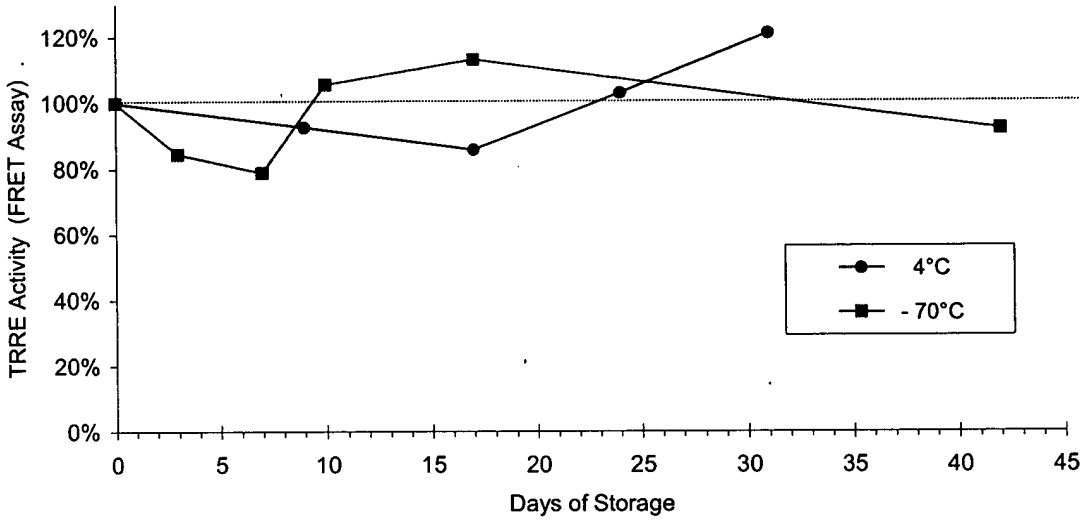
In this experiment, the amount of TRRE was titered out to determine the minimum effective dose. The enzyme was administered in a volume of 110 μL at one hour before the LPS challenge. The graph shows the mortality in each of the treatment groups in the study 24 hours after injection of the LPS challenge.

The protective effect of TRRE is dose-dependent. The LPS/galactosamine challenge was invariably fatal in mice treated only with saline control. At a single dose of 30,000 FU, MP8 was able to protect a proportion of the challenged subjects. At 300,000 FU, MP8 was completely protective.

In other experiments, it was shown that TRRE is also protective when administered simultaneously with the LPS, rather than in advance.

Eff ct of long-t rm storag on proteolytic activity

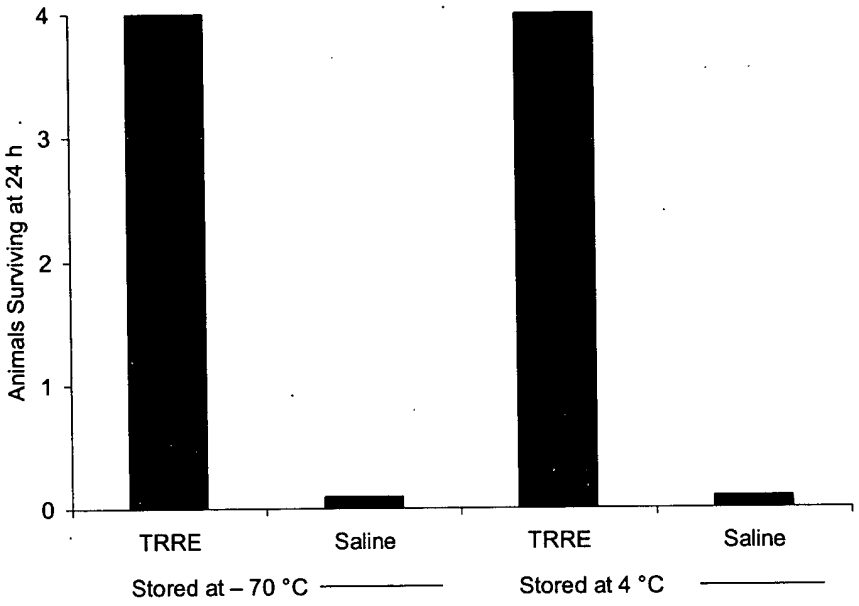
The stability of TRRE was determined by storing aliquots of MP8 at 4°C or -70°. Aliquots were taken out periodically to determine TRRE activity in the fluorescence resonance energy transfer peptide cleavage assay.



In its purified form, MP8 is as stable in a standard refrigerator as it is in deep freeze. There was no detectable loss of activity after a month of storage.

Protection against septic shock by stored TRRE

Stability of the clinical effect of TRRE was determined in the septic shock model. Female Balb/c mice were injected with 300,000 FU of MP8 that had been stored for 4 days at -70°C, or 7 days at 4°C. One hour later, the mice were challenged with 10 µg LPS and 7 mg galactosamine as before.



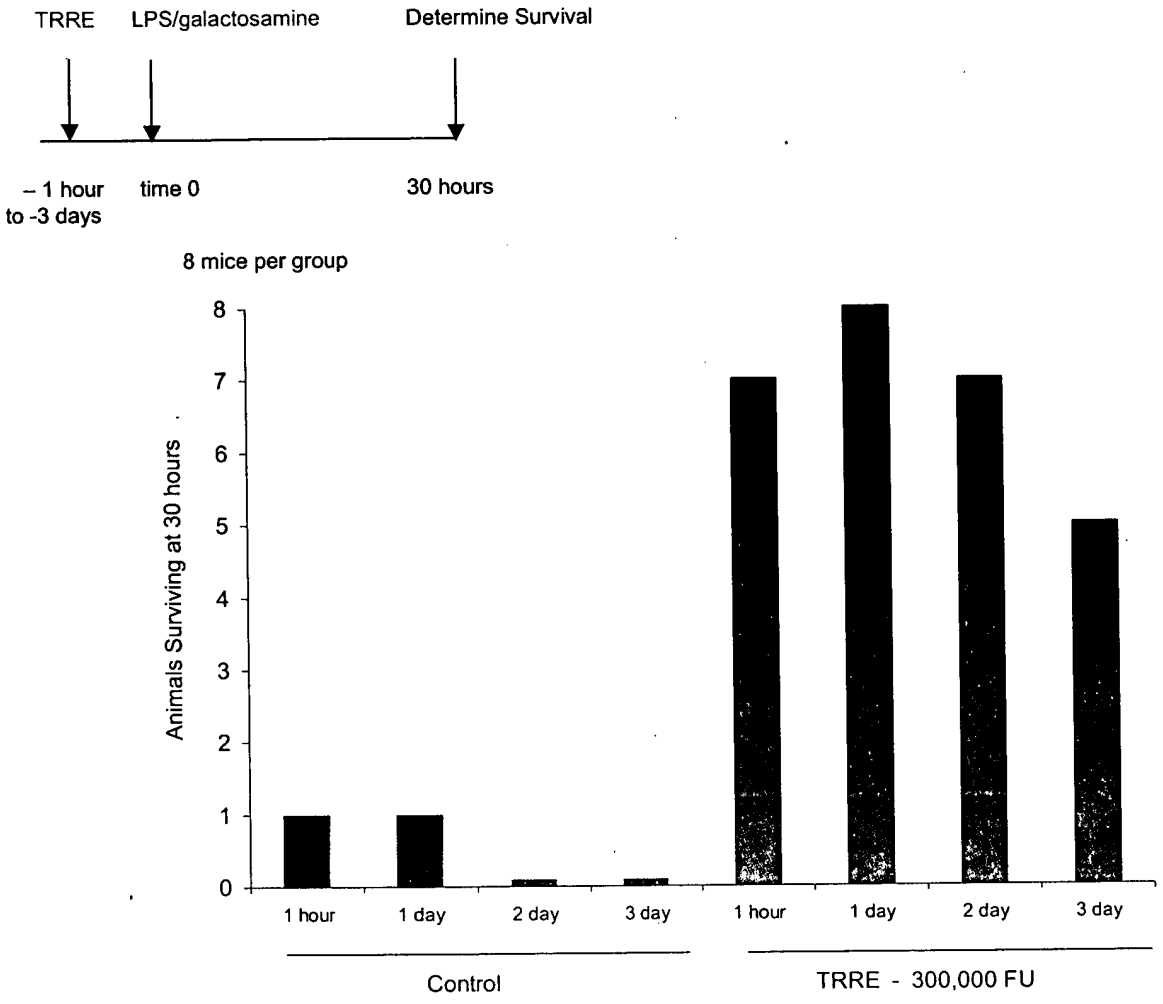
The data show that purified TRRE has a stable shelf life, and can be frozen without causing a loss in clinical efficacy.

P rsistence of TRRE in vivo

In this experiment, durability of TRRE *in vivo* was determined by treating animals with a *single dose* of MP8 (300,000 FU) up to three days in advance. The mice were then challenged with 10 μg LPS and 7 mg galactosamine in the usual fashion.

The data show that TRRE is able to protect most mice against LPS challenge up to 3 days after administration. This is either because TRRE has a long circulating half-life, or because an effect of the treatment (such as release of TNF receptor) persists in such a manner that TNF signal transduction is substantially reduced for several days.

Either way, this result means that frequent dosing with TRRE is not required for clinical efficacy.



TRRE treats experimentally induced arthritis

The potential clinical effectiveness of TRRE was assessed in collagen-induced arthritis, an animal model for rheumatoid arthritis.

Two separate protocols were used. In one experiment, the animals were treated daily with 300,000 FU of TRRE, simultaneously with administration of the disease agent. Eight out of 9 control mice were affected, but TRRE treated mice showed no joint swelling or other signs of arthritis.

In a second experiment, TRRE was administered to animals with established collagen-induced arthritis. TRRE effectively decreased the joint swelling in established disease when administered at a dose as low as 30,000 FU.

The animal model for rheumatoid arthritis

Collagen-induced arthritis is a standard model for evaluating potential therapeutic agents for rheumatoid arthritis.

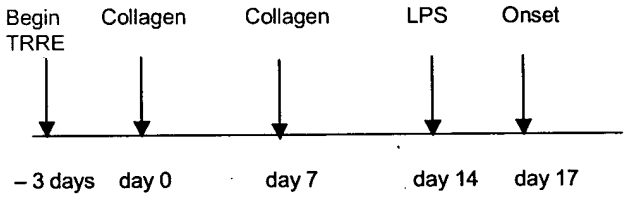
Procedure: Arthritis was induced in 7-9 week old female DBA/1LacJ mice by immunization with collagen. On day 0, the mice were injected at the base of the tail with 100 μg bovine type II collagen in complete Freund's adjuvant. On day 7, mice were boosted with an intraperitoneal injection of 100 μg collagen. To enhance and synchronize synovitis, the mice were injected subcutaneously on day 14 with 100 μg of lipopolysaccharide (LPS). Joint swelling was monitored in a blinded fashion by measuring the diameter in all 4 paws and both ankles using a constant pressure gauge. Arthritis Index was also determined on the following scale, and summed for all extremities. 0 ≡ normal; 1 ≡ one digit swollen; 2 ≡ more than one digit swollen; 3 ≡ joint distortion; 4 ≡ ankylosis.

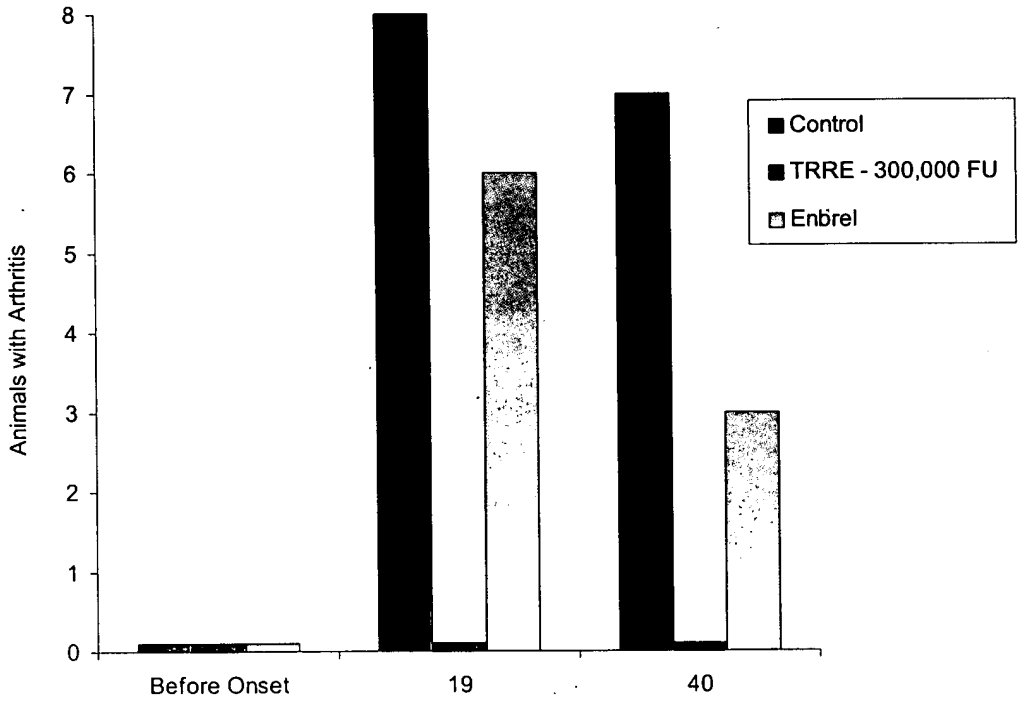
In the first experiment, TRRE clone MP8 was administered as a prophylactic agent, before there were signs of arthritis. In the second experiment, MP8 was administered to animals with established disease. Both experiments were conducted under contract with Calvert Preclinical Services, Inc., an independent laboratory.

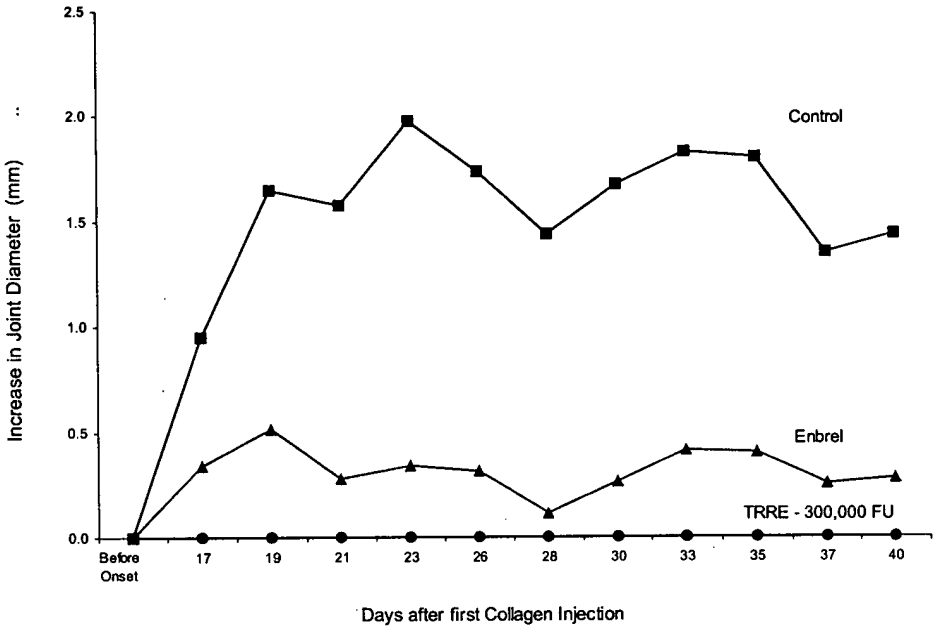
TRRE protects against collag n-induc d arthritis

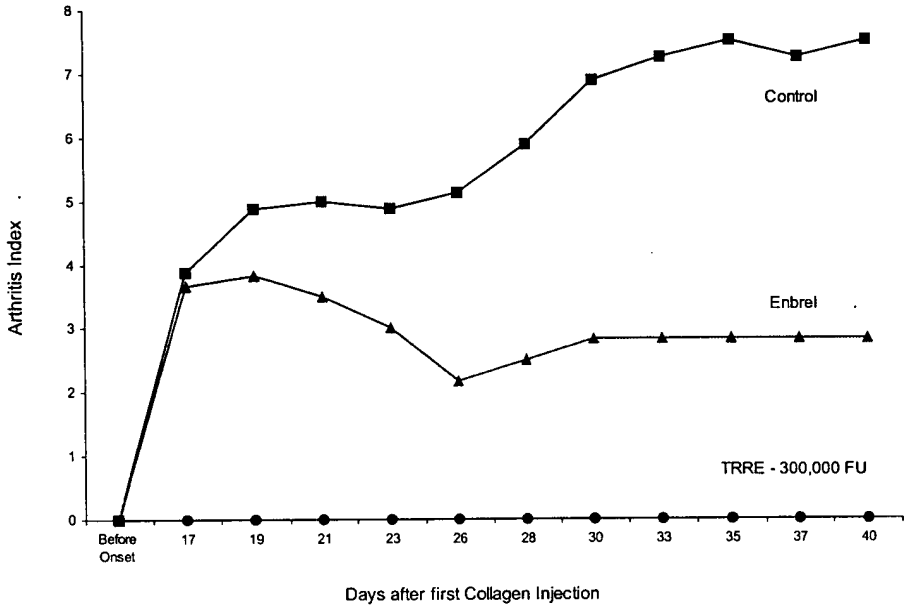
In the first experiment, mice were immunized with collagen on days 0 and 7, and then injected with LPS on day 14 (10 in each group). Beginning 3 days before the first collagen injection, they were treated daily with 300,000 FU of MP8, a scaled dose of *Enbrel*®, or saline control.

As is typical in this model, not all animals respond to the collagen challenge. In this experiment, 8 out of the 10 control animals showed signs of arthritis. However, none of the animals treated with TRRE were affected.









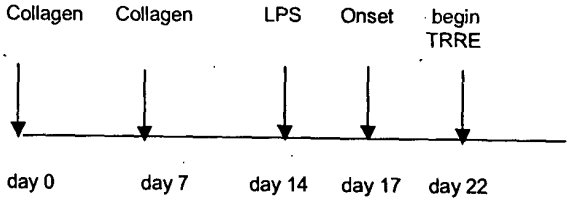
These graphs show the average increase in joint swelling and arthritis index measured for the animals in each group. When TRRE was given prophylactically, it prevented the animals from showing any measurable signs of the disease.

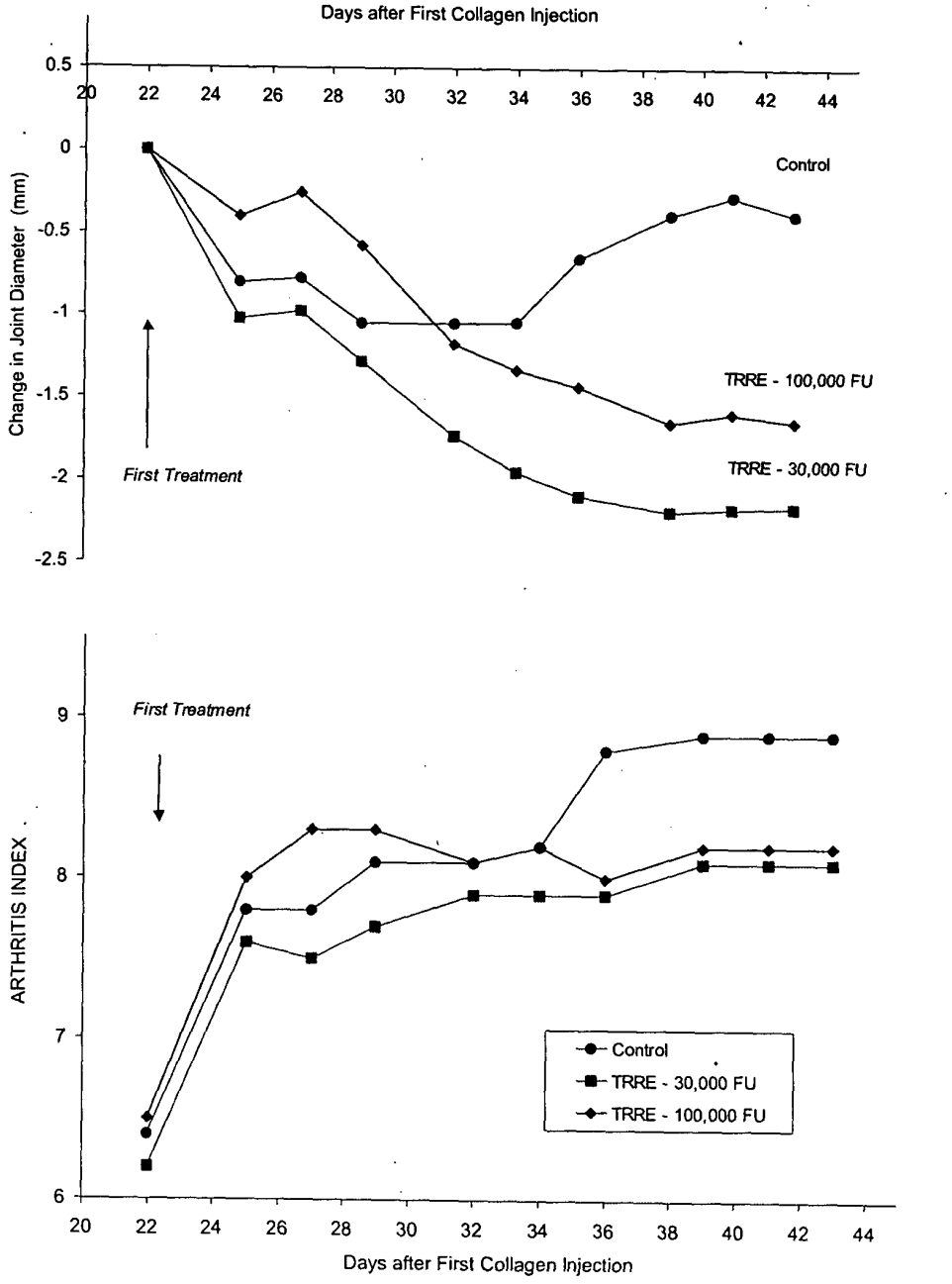
TRRE prevents progression of establish d arthritis

In the second experiment, TRRE clone MP8 was tested for its ability to treat established disease. This protocol is closer to the clinical situation in rheumatoid arthritis, where patients are treated after the onset of inflammatory synovitis.

Procedure: Mice were immunized with collagen on days 0, and 7, and then boosted with LPS on day 14. Treatment with TRRE was initiated on day 22 when arthritis was well established. At that time, animals with arthritis were randomized into three groups, and unaffected animals were excluded. The three affected groups were then treated for 18 consecutive days with saline control, or with MP8 at either of two different doses.

Results are shown on the next page.

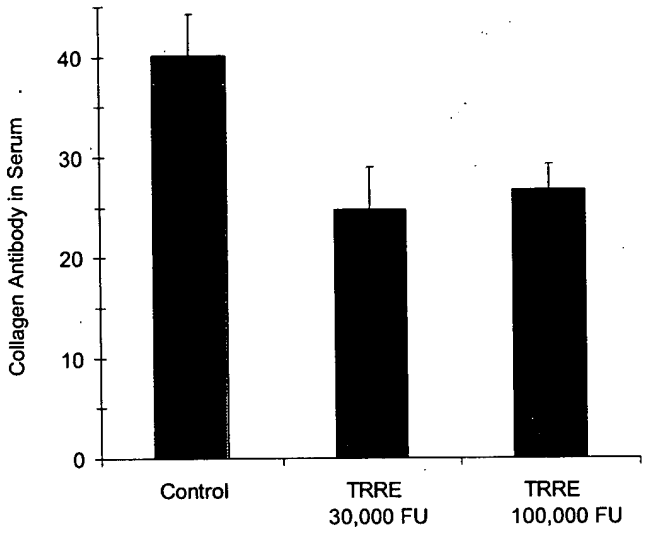

Collagen
Collagen
LPS
Onset
begin TRRE
day 0
day 7
day 14
day 17
day 22


Days after First Collagen Injection
Change in Joint Diameter (mm)
0.5
0
-0.5
-1
-1.5
-2
-2.5
20
22
24
26
28
30
32
34
36
38
40
42
44
Control
TRRE - 100,000 FU
TRRE - 30,000 FU
First Treatment
ARTHRITIS INDEX
9
8
7
6
First Treatment
Control
TRRE - 30,000 FU
TRRE - 100,000 FU
Days after First Collagen Injection

The results show a highly significant reduction in swelling in the affected joints of the two MP8-treated groups compared with control (n = 10 in each group; $p < 0.001$ at the end of the experiment, 1-tailed Student's *t*-test).

The control animals had little change in joint swelling after daily treatment was commenced at day 22. In contrast, the animals treated with TRRE at either dose showed substantial regression of disease.

Serum obtained at the end of the experiment was assayed for anti-type II collagen antibody levels by ELISA.



The groups treated with low or high doses of TRRE had levels of pathogenic antibody that were almost half the control (both $p < 0.02$). The group treated at the higher dose of TRRE also had lower severity of inflammatory synovitis as determined by histopathology at the end of the experiment.

No toxicity was detected in the MP8 treated groups.

The experiments described in this section show that systemic administration of TRRE is both safe and effective in the treatment of experimentally induced arthritis — even in established disease. TRRE reduces the level of circulating autoantibody, and prevents or reverses joint swelling.

TRRE is effective in th r inflammatory dis ase models

In the carrageenan-induced paw edema model, systemically administered TRRE was at least as effective as orally administered indomethacin in protecting against cytokine-mediated fluid accumulation.

In Experimental Autoimmune Encephalomyelitis, an animal model for Multiple Sclerosis, TRRE was found to delay emergence of symptoms from 12 days to 18 days, and lowered disease severity by about 3-fold.

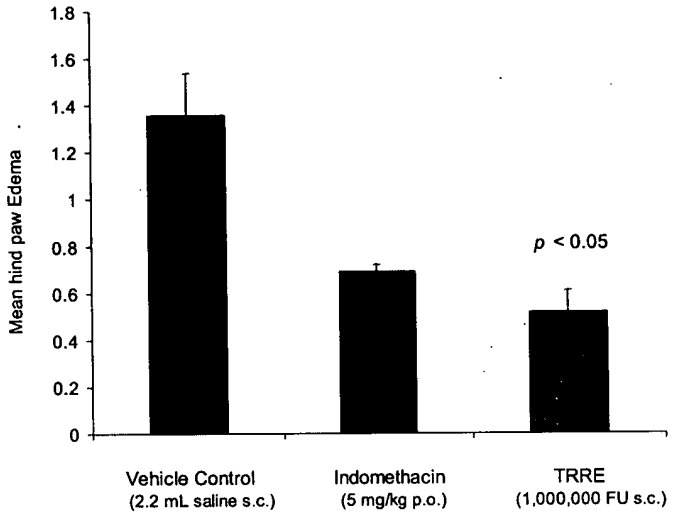
In an ovalbumin model for asthma, TRRE modulated the profile of inflammatory cells at the disease site.

Further experiments were performed to determine whether the beneficial effects of TRRE extend beyond arthritis. The disease models shown in this section demonstrate that the anti-inflammatory effects of TRRE can be effective in a variety of important clinical conditions in which inflammation plays a pathological role. All these experiments were performed under contract with Calvert Preclinical Services Inc.

TRRE inhibits carrageenan-induc d edema

TRRE clone MP8 was tested for its ability to inhibit carrageenan-induced paw swelling, in comparison with indomethacin, a small molecule anti-inflammatory drug.

Procedure: Male Sprague Dawley rats were randomized by weight into three treatment groups. The animals were administered MP8 or control solution subcutaneously, or indomethacin by mouth. One hour later, they were injected in the left hind paw with a sterile solution of 1% carrageenan suspension in water, in order to induce swelling. Three hours later, the volume of the injected paw was measured by water displacement (mean ± SEM; 10 animals per group).

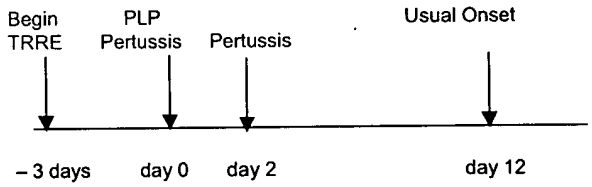


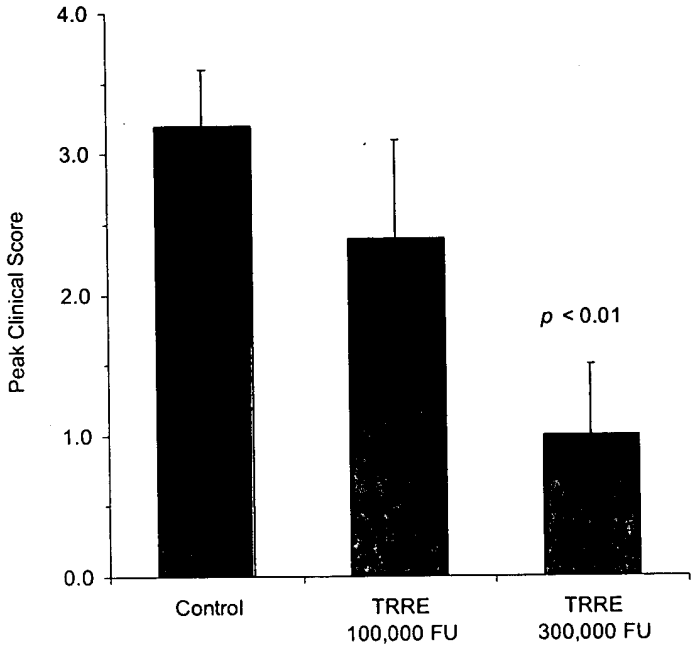
TRRE inhibited edema formation by 62% ($p \leq 0.05$). In comparison, the indomethacin inhibited edema formation by only 49%.

TRRE prevents development of Multipl Sclerosis

TRRE clone MP8 was also tested for its ability to protect against Experimental Autoimmune Encephalomyelitis (EAE), an established animal model for Multiple Sclerosis.

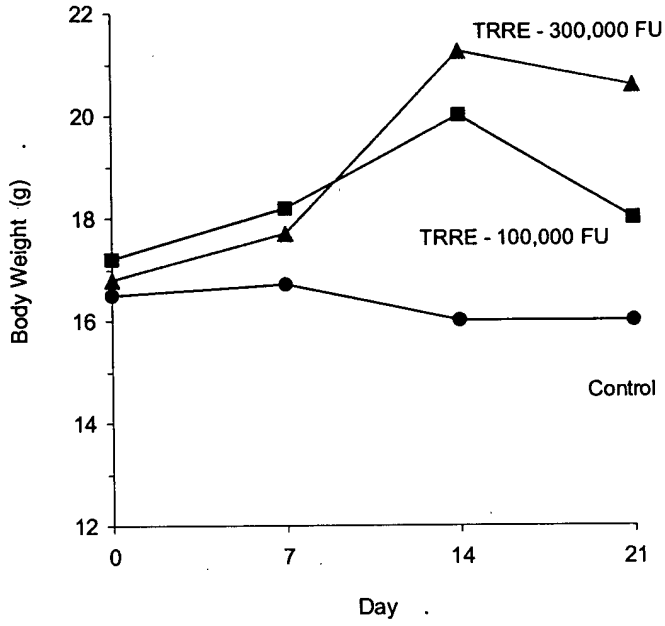
Procedure: Female SJL/J mice (6 weeks old) were randomized into 3 groups of 10. 2 mL of Myelin Proteolipid Peptide (PLP) was emulsified in 3 mL of Complete Freund's Adjuvant containing an additional 20 mg of *M. tuberculosis* H37Ra. On day 0, mice were immunized subcutaneously in the base of the tail and footpad with a total of 60 µg PLP. They were also given 400 mg pertussis toxin i.p. on days 0 and 2. MP8 or saline control was administered s.c. every day from day –3 to day 20. Progression of the disease was measured up to day 21 on the following scale: 0 ≡ normal; 1 ≡ limp tail or hind limb weakness; 2 ≡ both limp tail and hind limb weakness; 3 ≡ partial hind limb paralysis; 4 ≡ complete hind limb paralysis; 5 ≡ moribund or terminated.





TRRE had four clinically important effects.

- It completely prevented the disease from appearing in a proportion of animals
- In the animals that were affected, TRRE substantially delayed the onset of symptoms
- It reduced the severity of the disease by over 3-fold
- Treated animals continued to show normal weight gain

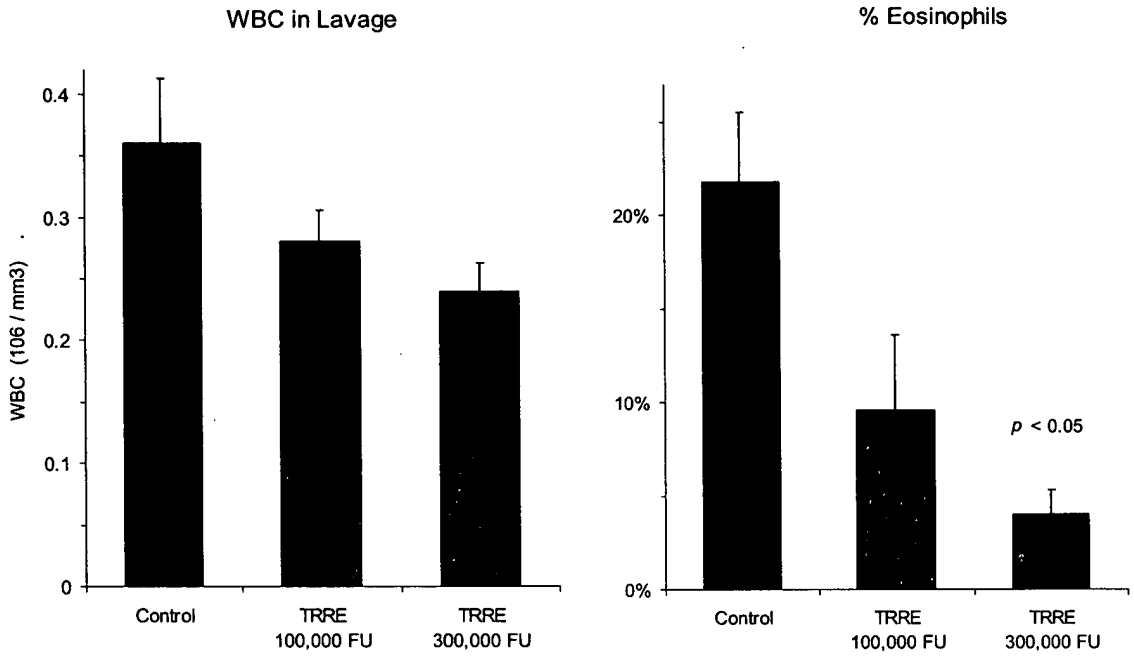


Treatment	Incidence of Disease	Day of Onset (± SEM)	Peak Clinical Score (± SEM)
Saline Control	7 out of 7	12.7 ± 0.6	3.2 ± 0.4
TRRE - 100,000 FU	5 out of 7	18.0 ± 0.8 [a]	2.4 ± 0.7
TRRE - 300,000 FU	3 out of 7	18.0 ± 2.5 [a]	1.0 ± 0.5 [b]
		[a] $p < 0.001$	[b] $p < 0.01$

TRRE limits th c llular effect of experim ntally-induced Asthma

In a further animal model for inflammation, TRRE clone MP8 was tested for its ability to modulate the pathology associated with experimentally induced Asthma.

Procedure: Mice were sensitized on Days 0, 7, and 14 with 10 µg ovalbumin in 1% aluminum hydroxide. On Day 21, the mice were challenged with the allergen in aerosol form (5% wt/vol in saline). Treatment with TRRE or control was administered 1 h before the aerosol challenge, and 24 h and 48 h afterwards. On Day 24 (72 h after the challenge), lungs were lavaged under anesthesia with 2 x 0.5 mL buffer to recover cells in the alveolar fluid.



The results show that TRRE reduces the number and nature of the white blood cells migrating into the alveolar fluid.

Patent Prot ction for TRRE

Patent protection for TRRE is being obtained worldwide. The enzyme family has been patented as both compositions of matter, and use for cleaving the TNF receptor. The important cDNA sequences are novel, and divisional applications are pending for recombinant production, and use of the enzymes for treating inflammatory conditions. Other unpublished patent applications provide protection for more recent developments.

8

Patent family for Native TNF Receptor Releasing Enzyme

The first patent family protects isolated TRRE in a form present in human cells that naturally express it. These patents are licensed exclusively to Meyer Pharmaceuticals from the University of California. Divisional applications cover methods of identifying TRRE, and the use of TRRE for affecting TNF receptor transduction and screening for small-molecule drugs.

Jurisdiction	Serial No.	Effective Filing Date	Status
US	60/030,761	Nov 6/96	Provisional
US	6,569,664	Nov 5/97	Issued
US	*6,573,062*	Nov 5/97	Issued
US	*(Divisionals)*		Pending
PCT	WO 98/20140	Nov 5/97	National Stage
Australia	AU 744,873	Nov 5/97	Granted
Brazil	PI 9712900-3	Nov 5/97	Pending
Canada	2,270,898	Nov 5/97	Pending
Europe	EP 938548-A1	Nov 5/97	Pending
Hong Kong	00101273.6	Nov 5/97	Pending
Indonesia	W-990460	Nov 5/97	Pending
Israel	129787	Nov 5/97	Pending
Japan	10-521643	Nov 5/97	Pending
South Korea	99-7003993	Nov 5/97	Pending
Mexico	994209	Nov 5/97	Pending
New Zealand	NZ 335864	Nov 5/97	Granted
Norway	P19992187	Nov 5/97	Pending
Singapore	SG 65360	Nov 5/97	Granted

US006569664B1

(12) **United States Patent**
Gatanaga et al.

(10) **Patent No.: US 6,569,664 B1**
(45) **Date of Patent: May 27, 2003**

(54) **NATIVE TNF RECEPTOR RELEASING ENZYME**

(75) Inventors: **Tetsuya Gatanaga**, Irvine, CA (US); **Gale A. Granger**, Laguna Beach, CA (US)

(73) Assignee: **The Regents of the University of California**, Oakland, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/964,747**

(22) Filed: **Nov. 5, 1997**

Related U.S. Application Data

(60) Provisional application No. 60/030,761, filed on Nov. 6, 1996.

(51) Int. Cl.[7] **C12N 9/64**; C07K 1/14; C07K 1/18; C07K 1/26

(52) U.S. Cl. **435/226**; 435/68.1; 530/412; 530/416; 530/417

(58) Field of Search 435/226; 530/412, 530/413, 417; 424/94.63

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,340,586	A	7/1982	Bekierkunst et al.	424/282.1
4,695,590	A	9/1987	Lippman	514/724
4,959,353	A	9/1990	Brown et al.	514/12
4,983,586	A	1/1991	Bodor	514/58

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

EP	211 077	5/1993
EP	568 925	11/1993
EP	623 676	11/1994
EP	657 536	6/1995
EP	418 014	12/1995
WO	WO 90/11287	10/1990
WO	WO 90/14363	11/1990
WO	WO 91/04014	4/1991
WO	WO 93/07286	4/1993
WO	WO 93/20186	10/1993
WO	WO 94/22309	10/1994
WO	WO 95/09913	4/1995
WO	WO 95/13365	5/1995
WO	WO 95/31544	11/1995
WO	WO 95/33051	12/1995
WO	WO 96/01642	1/1996

OTHER PUBLICATIONS

Katsura, K., et al., 1996, Biochemical and Biophysical Research Communications, vol. 222, Identification of the proteolytic enzyme which cleaves human p75 TNF receptor in vitro, pp. 298–302.*

Solorzano, C.C., et al., 1997, The Journal of Immunology, vol. 97, "Involvement of 26-kDa cell-associated TNF-alpha in experimental hepatitis and exacerbation of liver injury with a matrix metalloproteinase inhibitor", pp. 414–419.*

Wu, A.J., et al., 1997, Journal of Cellular Physiology, vol. 171, "Modulation of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) by interferon-gamma in a human salifary gland cell line", pp. 117–124.*

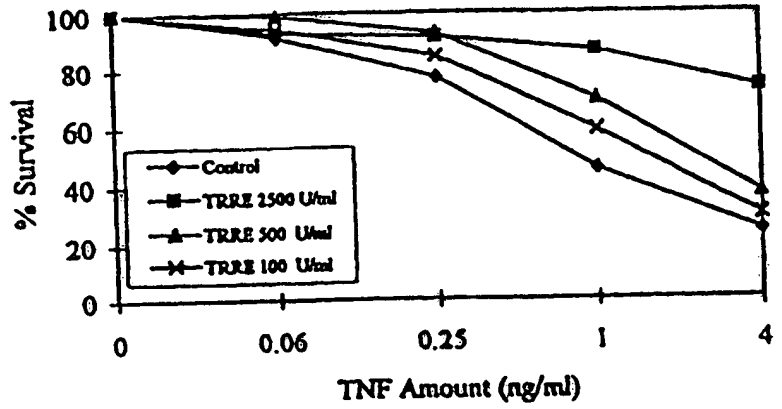
(List continued on next page.)

Primary Examiner—Ponnathapu Achutamurthy
Assistant Examiner—William W. Moore
(74) *Attorney, Agent, or Firm*—Bozicevic, Field and Francis LLP; Carol L. Francis

(57) **ABSTRACT**

The present invention relates to methods of regulating TNF activity indirectly by regulating the activity or concentration of TNF receptor releasing enzyme (TRRE). Preferably, the TRRE activity is regulated local to the site of the condition to be treated. In the case of diseases associated with elevated levels of TNF, such as rheumatoid arthritis, TRRE is administered to the site of inflammation in an amount sufficient to decrease the local levels of TNF. In the case of diseases, such as cancer, that benefit from increased levels of TNF, the level of TRRE is decreased at the disease site.

28 Claims, 13 Drawing Sheets



U.S. Patent 6,593,456

Native TNF receptor releasing enzyme

Issued claims

1. An isolated composition comprising a TNF receptor releasing enzyme, obtainable by a process comprising:
   a) stimulating THP-1 cells with phorbol myristate acetate;
   b) harvesting culture medium from the stimulated cells; and
   c) isolating the enzyme from the harvested medium;
   wherein the enzyme causes cleaving and release of TNF receptor expressed on a cell surface.

2. The composition of claim 1, isolated by a method comprising ion exchange chromatography and separation by molecular weight.

3. The composition of claim 1, obtained by separating a polypeptide having an apparent molecular weight by SDS polyacrylamide gel electrophoresis of about 120,000.

4. The composition of claim 3, which is at least about 95% homogeneous when analyzed by SDS polyacrylamide gel electrophoresis.

5. The composition of claim 1, obtained by separating a polypeptide having an apparent molecular weight by SDS polyacrylamide gel electrophoresis of about 60,000.

6. The enzyme composition of claim 5, which is at least about 95% homogeneous when analyzed by SDS polyacrylamide gel electrophoresis.

7. The composition of claim 1, obtained by separating a polypeptide having an apparent molecular weight by SDS polyacrylamide gel electrophoresis of about 37,000.

8. The composition of claim 7, which is at least about 95% homogeneous when analyzed by SDS polyacrylamide gel electrophoresis.

9. The composition of claim 1, having enzymatic activity that is inhibitable by a metalloprotease inhibitor but not by a serine protease inhibitor or a cysteine protease inhibitor.

10. The composition of claim 1, having enzymatic activity that is enhanced in the presence of $Ca.^{++}$ and is diminished in the presence of phenanthroline.

11. A method of obtaining th composition of claim 1, comprising:

a) culturing a cell expressing an enzym that causes cl aving and rel as of TNF receptor expressed on a cell surface, said culturing being under conditions to cause th r l ase of TNF receptor releasing enzyme activity into the medium;

b) harvesting medium from the cultured cell; and,

c) isolating the composition from the culture medium.

12. The method of claim 11, wherein the isolating comprises ion exchange chromatography and separation by molecular weight.

13. The method of claim 11, wherein the composition has been obtained from THP-1, U-937, HL-60, ME-180, MRC-5, Raji, or K-562 cells or normal human monocytes.

14. A method for decreasing signal transduction from TNF into a cell, comprising contacting the cell with the composition of claim 1, under conditions where the composition causes cleaving and release of TNF receptors from the cell surface.

15. The composition of claim 1, which is obtainable from the supernatant of cultured THP-1 cells upon induction with PMA, without de novo protein synthesis.

16. The composition of claim 1, which is obtainable from the supernatant of cultured THP-1 cells upon induction by IL-10 or epinephrine.

17. The composition of claim 1, which causes cleaving and release of the human p75 TNF receptor.

18. The composition of claim 1, which causes cleaving and release of the human p55 TNF receptor.

19. The composition of claim 1, which has an apparent molecular weight of 66-200 kDa as determined by gel filtration chromatography on Sephadex G-150 at pH 7.0.

20. The composition of claim 1, which has an apparent molecular weight of about 150 kDa as determined by gel filtration chromatography on Sephadex G-150 at pH 7.0.

21. The composition of claim 1, which migrates as a single band when subject to polyacrylamide gel electrophoresis in non-denaturing conditions.

22. The composition of claim 1, which is at least 80% pure (wt/wt protein).

23. Th composition of claim 1, wherein TNF receptor releasing activity as measured by cleavage of p75 TNF receptor from isolat d cells is at least 5,000 U/mL.

24. The composition of claim 1, wherein TNF receptor releasing activity as measured by cleavage of p75 TNF receptor from isolated cells is at least 15,000 U/mL.

25. The composition of claim 1, wherein TNF receptor releasing activity as measured by cleavage of p75 TNF receptor from isolated cells is at least 40,000 U/mL.

26. The composition of claim 1, obtained according to the method of claim 11.

27. The method of claim 14, wherein the composition has been obtained according to the method of claim 11.

28. The method of claim 14, wherein the composition has been obtained from THP-1 cells.

Patent family for s qu nces of the TRRE cl n s

The second patent family covers the TRRE cDNA clones according to their nucleotide and protein sequence. These patents are also licensed exclusively to Meyer Pharmaceuticals. The metalloprotease clones MP7 and MP8 have novel sequences with no previously known function. Accordingly, these clones and their functional variants are protectable as compositions of matter. Claims are also pending for the use of these clones in the treatment of inflammatory conditions such as arthritis.

Jurisdiction	Serial No.	Effective Filing Date	Status
US	6,593,456	May 14/98	Issued
PCT	WO 99/58559	May 14/99	National Stage
US	*(National Stage)*	May 14/99	Pending
US	*(Divisionals)*	May 14/99	Pending
Argentina	P990102289	May 14/99	Pending
Malaysia	PI9901910	May 14/99	Pending
Taiwan	88107897	May 14/99	Pending
Australia	39960/99	May 14/99	Pending
Brazil	PI 9910458-0	May 14/99	Pending
Canada	2,328,133	May 14/99	Pending
Mainland China	1309709T	May 14/99	Pending
Europe	EP 1076710-A2	May 14/99	Pending
Israel	139566	May 14/99	Pending
India	PCT/20000650/CHE	May 14/99	Pending
Japan	2000-548361	May 14/99	Pending
South Korea	2000-7012726	May 14/99	Pending
Mexico	11011	May 14/99	Pending
New Zealand	507977	May 14/99	Pending
Russian Republic	2000131207	May 14/99	Pending
Singapore	2000 06550-8	May 14/99	Allowed
South Africa	ZA 2000/6475	May 14/99	Granted

U.S. Pat nt 6,593,456

Tumor necrosis factor receptor releasing enzyme
[Cloned TRRE]

Issued claims

1. An isolated polypeptide selected from the following:
   a) a protein with a complete amino acid sequence encoded in any of SEQ. ID NOs: 1, 5, 6, 8, 9, or 10;
   b) a fragment of said protein; and
   c) a fusion protein containing the protein or fragment according to a) or b);
   wherein the polypeptide causes TNF receptor to be released from cells expressing the receptor.

2. The polypeptide of claim 1, which is a protein with a complete amino acid sequence encoded in any of SEQ. ID NOs: 1, 5, 6, 8, 9, or 10.

3. The polypeptide of claim 1, comprising a fragment encoded within any of SEQ. ID NOs: 1, 5, 6, 8, 9, or 10, which causes TNF receptor to be cleaved and released from cells expressing said receptor.

4. The polypeptide of claim 3, comprising said fragment fused to another amino acid sequence.

5. The polypeptide of claim 1, comprising an amino acid sequence contained in any of SEQ. ID NOs: 151, 153, or 154.

6. The polypeptide of claim 1, comprising the complete amino acid sequence in any of SEQ. ID NOs: 151, 153, or 154.

7. The polypeptide of claim 1, which is a metalloproteinase.

8. The isolated polypeptide of claim 1, which cleaves TNF receptor from the surface of cells.

9. The isolated polypeptide of claim 1, which causes release of the human p55 TNF receptor from cells expressing the receptor.

10. The isolated polypeptide of claim 1, which causes release of the human p75 TNF receptor from cells expressing the receptor.

11. The polypeptide of claim 1, selected from the following:
    a) a protein with a complete amino acid sequence encoded in SEQ. ID NO: 1;
    b) a fragment of said protein; and
    c) a fusion protein containing the protein or fragment according to a) or b);
    wherein the polypeptide causes TNF receptor to be released from cells expressing the receptor.

12. Th polypeptid of claim 1, s l cted from the following:
    a) a protein with a compl t amino acid sequence encoded in SEQ. ID NO:5;
    b) a fragment of said protein; and
    c) a fusion protein containing the protein or fragment according to a) or b);
    wherein the polypeptide causes TNF receptor to be released from cells expressing the receptor.

13. The polypeptide of claim 1, selected from the following:
    a) a protein with a complete amino acid sequence encoded in SEQ. ID NO: 6;
    b) a fragment of said protein; and
    c) a fusion protein containing the protein or fragment according to a) or b);
    wherein the polypeptide causes TNF receptor to be released from cells expressing the receptor.

14. The polypeptide of claim 1, selected from the following:
    a) a protein with a complete amino acid sequence encoded in SEQ. ID NO: 8;
    b) a fragment of said protein; and
    c) a fusion protein containing the protein or fragment according to a) or b);
    wherein the polypeptide causes TNF receptor to be released from cells expressing the receptor.

15. The polypeptide of claim 1, selected from the following:
    a) a protein with a complete amino acid sequence encoded in SEQ. ID NO:9;
    b) a fragment of said protein; and
    c) a fusion protein containing the protein or fragment according to a) or b);
    wherein the polypeptide causes TNF receptor to be released from cells expressing the receptor.

16. The polypeptide of claim 1, selected from the following:
    a) a protein with a complete amino acid sequence encoded in SEQ. ID NO: 10;
    b) a fragment of said protein; and
    c) a fusion protein containing the protein or fragment according to a) or b);
    wherein the polypeptide causes TNF receptor to be released from cells expressing the receptor.

17. An isolated polypeptide selected from the following:
    a) a protein with a complete amino acid sequence encoded in SEQ. ID NO:8;
    b) a fragment of said protein; and
    c) a fusion protein containing the protein or fragment according to a) or b);
    wherein the polypeptide has metalloproteinase activity.

18. An isolated polypeptide selected from the following:
    a) a protein with a complete amino acid sequence encoded in SEQ. ID NO:9;
    b) a fragment of said protein; and
    c) a fusion protein containing the protein or fragment according to a) or b);
    wherein the polypeptide has metalloproteinase activity.

19. A pharmaceutical composition, comprising the polypeptide of claim 1, in a suitable excipient.

20. A pharmaceutical composition, comprising the polypeptide of claim 17, in a suitable excipient.

21. A pharmaceutical composition, comprising the polypeptide of claim 18, a suitable excipient.

22. A method of causing enzymatic release of TNF receptor from a cell, comprising contacting the cell with a polypeptide according to claim 1.

23. A method of causing enzymatic release of TNF receptor from a cell, comprising contacting the cell with a polypeptide according to claim 11.

24. A method of causing enzymatic release of TNF receptor from a cell, comprising contacting the cell with a polypeptide according to claim 12.

25. A method of causing enzymatic release of TNF receptor from a cell, comprising contacting the cell with a polypeptide according to claim 13.

26. A method of causing enzymatic release of TNF receptor from a cell, comprising contacting the cell with a polypeptide according to claim 14.

27. A method of causing enzymatic release of TNF receptor from a cell, comprising contacting the cell with a polypeptide according to claim 15.

28. A method of causing enzymatic release of TNF receptor from a cell, comprising contacting the cell with a polypeptide according to claim 16.

29. A method of causing enzymatic release of TNF receptor from a cell, comprising contacting the cell with a polypeptide according to claim 17.

30. A method of causing enzymatic release of TNF receptor from a cell, comprising contacting the cell with a polypeptide according to claim 18.

31. A method of altering signal transduction from TNF into a cell, comprising contacting the cell with a polypeptide according to claim 1.

Background Articles

Tumor Necrosis Factor and the TNF Receptor

1. Granger GA, Shacks SJ, Williams TW, Kolb WP. Lymphocyte in vitro cytotoxicity: specific release of lymphotoxin-like materials from tuberculin-sensitive lymphoid cells. Nature 221:1155, 1969.

2. Gatanaga T, Hwang CD, Kohr W, Cappuccini F, Lucci JA 3rd, Jeffes EW, Lentz R, Tomich J, Yamamoto RS, Granger GA. Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for tumor necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients. Proc Natl Acad Sci USA 87:8781, 1990.

3. Smith CA, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D, Goodwin RG. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science 248:1019, 1990.

4. Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko V, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. [Review] Annu Rev Immunol 17:331, 1999.

5. McDermott MF. TNF and TNFR biology in health and disease. [Review] Cell Mol Biol 4:619, 2001.

TNF and IL-6 Receptor shedding

6. Grosen EA, Granger GA, Gatanaga M, Ininns EK, Hwang C, DiSaia P, Berman M, Manetta A, Emma D, Gatanaga T. Measurement of the soluble membrane receptors for tumor necrosis factor and lymphotoxin in the sera of patients with gynecologic malignancy. Gynecol Oncol 50:68, 1993.

7. Gatanaga M, Grosen EA, Burger RA, Granger GA, Gatanaga T. Release of soluble TNF/LT receptors from a human ovarian tumor cell line (PA-1) by stimulation with cytokines in vitro. Lymphokine Cytokine Res. 12:249, 1993.

8. Katsura K, Park M, Gatanaga M, Yu EC, Takishima K, Granger GA, Gatanaga T. Identification of the proteolytic enzyme which cleaves human p75 TNF receptor in vitro. Biochem Biophys Res Commun. 222:298, 1996.

9. Shedding of tumor necrosis factor receptors by activated human neutrophils. Porteu F, Nathan C. J Exp Med 172:599, 1990.

10. Tumor necrosis factor (TNF)-dependent shedding of the p55 TNF receptor in a baboon model of bacteremia. Redl H, Schlag G, Adolf GR, Natmessnig B, Davies J. Related Articles, Domains Infect Immun. 63:297, 1995.

11. Mullberg J, Durie FH, Otten-Evans C, Alderson MR, Rose-John S, Cosman D, Black RA, Mohler KM. A metalloprotease inhibitor blocks shedding of the IL-6 receptor and the p60 TNF receptor. J Immunol 155:5192, 1995.

12. Arribast J, Lopez-Casillas F, Massague J. Role of the juxtamembrane domains of the transforming growth factor-α precursor and the β-amyloid precursor protein in regulated ectodomain shedding. J Biol Chem 272:17160, 1997.

13. Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC, Russell WE, Castner BJ, Johnson RS, Fitzner JN, Boyce RW, Nelson N, Kozlosky CJ, Wolfson MF, Rauch CT, Cerretti DP, Paxton RJ, March CJ, Black RA. An essential role for ectodomain shedding in mammalian development. Science 282:1281, 1998.

14. Althoff K, Mullberg J, Aasland D, Voltz N, Kallen K-J, Grotzinger J, Rose-John S. Recognition sequences and structural elements contribute to shedding susceptibility of membrane proteins. Biochem J 353:663, 2001.

Other metalloproteases

15. Rosendahl MS, Ko SC, Long DL, Brewer MT, Rosenzweig B, Hedl E, Anderson L, Pyle SM, Moreland J, Meyers MA, Kohno T, Lyons D, Lichenstein HS. Identification and characterization of a pro-tumor necrosis factor-α-processing enzyme from the ADAM family of zinc metalloproteases. J Biol Chem 272:24588, 1997.

16. Roghani M, Becherer JD, Moss ML, Atherton RE, Erdjument-Bromage H, Arribas J, Blackburn RK, Weskamp G, Tempst P, Blobel CP. Metalloprotease-disintegrin MDC9: Intracellular maturation and catalytic activity. J Biol Chem 274:3531, 1999.

Animal models for inflammatory disease

17. Morrison DC, Silverstein R, Bright SW, Chen TY, Flebbe LM, Lei MG. Monoclonal antibody to mouse lipopolysaccharide receptor protects mice against the lethal effects of endotoxin [Septic shock model]. J Infect Dis 162:1063, 1990.

18. Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B. Immunization against heterologous type II collagen induces arthritis in mice [Collagen induced arthritis model]. Nature 283:666, 1980.

19. Williams RO, Feldmann M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci USA 89:9784, 1992.

20. Gerlag DM, Ransone L, Tak PP, Han Z, Palanki M, Barbosa MS, Boyle D, Manning AM, Firestein GS. The effect of a T cell-specific NF-κB inhibitor on in vitro cytokine production and collagen-induced arthritis. J Immunol 165:1652, 2000.

21. Feige U, Hu YL, Gasser J, Campagnuolo G, Munyakazi L, Bolon B. Anti-interleukin-1 and anti-tumor necrosis factor-alpha synergistically inhibit adjuvant arthritis in Lewis rats. Cell Mol Life Sci 57:1457, 2000.

22. Winter CA, Risley EA, Nuss GW. Carrageenan-induced edema in the hind paw of the rat as an assay for antiinflammatory drugs [Edema model]. Proc Soc Exp Biol Med 111:544, 1962.

23. Brown AM, McFarlin DE. Relapsing experimental allergic encephalomyelitis in the SJL/J mouse [Multiple Sclerosis model]. Lab. Invest. 45:278, 1981.

Biological therapy for inflammatory disease

24. Feldmann M, Maini RN. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? [Review] Annu Rev Immunol 19:163, 2001.

25. Taylor PC. Anti-tumor necrosis factor therapies. [Review] Curr Opin Rheumatol 13:164, 2001.

26. Garrison L, McDonnell ND. Etanercept [*Enbrel*®]: therapeutic use in patients with rheumatoid arthritis. Ann Rheum Dis. 58 Suppl 1:165, 1999.

27. Harriman G, Harper LK, Schaible TF. Summary of clinical trials in rheumatoid arthritis using infliximab [*Remicade*®], an anti-TNFalpha treatment. Ann Rheum Dis. 58 Suppl 1:I61, 1999.

28. Nishimoto N, Sasai M, Shima Y, Nakagawa M, Matsumoto T, Shirai T, Kishimoto T, Yoshizaki K. Improvement in Castleman's disease by humanized anti-interleukin-6 receptor antibody therapy. Blood 95:56, 2000.

29. Van de Putte LBA, Atkins C, Malaise M, Sany J, Russell AS, van Riell PLC, Settas L, Bijlsma JW, Todesco S, Dugados M, Nash P, Walter N, Kaul M, Kupper H. Efficacy and safety of adalimumab (D2E7): Six month results from a Phase III study. Annu Eur Congr Rheumatol. FRI0027, 2002.